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FIBRINOLYSIS INDUCED BY BRAIN EXTRACTS.

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In a previous paper from this Institute (Fantl and Simon, 1948) it was shown that under the influence of electro-convulsions a transient fibrinolytic activity appeared in human plasma whilst fibrinogen was not attacked. R. H. Mole (1948) reported recently that the fluidity of blood post mortem in cases of sudden death was also due to the dissolution of fibrin by an enzyme which did not split fibrinogen. Because of the significance which a specific fibrinolytic enzyme may have in the prevention of thrombo-embolic conditions, further studies of the mechanism of fibrinolysis were carried out.

It appears most likely that the appearance of the fibrinolytic enzyme occurs through a mechanism similar to that suggested by Christensen (1945), and Biggs, McFarlane and Pilling (1947), who assume that the enzyme precursor is a normal blood constituent, whilst a kinase is introduced following certain stimuli or pathology. A search for the source of the fibrinokinase was undertaken. The most consistent results were obtained by the use of extracts of human brain. A description of the experimental procedure follows.

EXPERIMENTAL.

Portions of human brain after removal of blood vessels and pia mater were rinsed with isotonic saline and ground with several lots of neutral acetone. The powder was introduced into ampoules which were sealed under vacuum and stored in a refrigerator. Prior to use a 6 per cent suspension was prepared by extraction with 0·85 per cent NaCl at 37° C. for 15 min. with occasional stirring. The suspension was centrifuged at 2700 G. for 10 min. and the supernatant fluid used. Little activity remained in the sediment. Plasma specimens were obtained from normal human donors by addition of 1 vol. 0·1 M sodium oxalate to 9 volumes of venous blood, and the cellular elements were removed by centrifugation. In one series of experiments 0·1 ml. oxalated plasma was added to

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1·3 ml. 0·85 per cent NaCl, and after addition of 0·1 ml. 0·25 M calcium chloride, containing 1 g. merthiolate in 400 ml., a small glass rod was inserted, the contents were mixed and incubated at 37° C. until the contents gelled. The fibrin clot was wound around the glass rod and 1 ml. of the brain extract added.

In a second series of experiments the fibrin was isolated on the glass rod in a similar manner to the first series, but before brain extract was added, the serum was removed and the clot rinsed with isotonic saline until soluble serum proteins were removed. This washed clot was incubated with brain extract.

A third series of experiments were carried out. These were similar to the first except that no calcium chloride was added and incubation was carried out for periods in which complete fibrinolysis occurred in the first series. Usually after 18 hours calcium chloride was added to these specimens.

TABLE I.—*Influence of Brain Extract on Fibrinolysis.*

Number of plasma specimens tested.	*Fibrinolysis occurring.*		*Fibrinogenolysis occurring* (3).
	(1) In presence of serum.	(2) In absence of serum.	
54	54	15	1*

* Tested in 10 cases only.

The results given in Table I (Col. 1) indicate that an extract of human brain contains a principle which, combined with a serum component, is capable of dissolving fibrin. This occurred in 4 to 16 hours at 37° C. That the process was dependent upon the presence of serum can be judged from Column 2. When the serum was removed fibrinolysis occurred in only approximately one-fourth of all cases tested. This result would indicate that the extracts contained little, if any, enzymes capable of attacking fibrin. They suggest that serum contains the precursor and brain the kinase. The last column, Table I, indicates that in contrast to the breakdown of fibrin, fibrinogen was not attacked in the majority of cases. These results could be verified with four different brain specimens. A fifth brain preparation, however, obtained from a child who died from leukaemia, was inactive. The following properties of the brain component were noticed. Heating to 70° C. for 15 min. destroyed the activity, as can be seen in Table II.

TABLE II.—*Heat Inactivation of Brain Fibrinokinase.*

Incubation temperature of brain extract.	Fibrinolysis time in hours.	Prothrombin time in seconds.
18° C.	18	17
70° C.	∞	20

In this instance the brain suspension was centrifuged at 540 G. In addition to the fibrinolysis tests the brain extracts were used as thromboplastin for the estimation of prothrombin. The results in the third column of Table II indicate that the thromboplastic property was not greatly influenced by heating.

These experiments suggest that the brain component required for fibrinolytic activity is different from the thromboplastin. This could be further substantiated by the use of heterologous brain.

TABLE III.—*Influence of Rabbit and Human Brain Extract on Human Fibrin and Fibrinogen.*

Brain extract used.	Fibrinolysis (1) In presence of serum.	Fibrinolysis (2) In absence of serum.	Fibrinogenolysis (3).
Human . . .	16	2 .	*Nil*
Rabbit . . .	3	2 .	,,

Columns (1) and (2)—16 plasma specimens tested.
Column (3) — 7 ,, ,, ,,

In Table III are shown the results obtained from plasma specimens which were tested with both human and rabbit brain extract. It is quite evident that in the majority of cases there was a marked species specificity with regard to brain fibrinokinase. Although in some cases both brain extracts were capable of dissolving isolated fibrin (Column 2), in no case was fibrinogenolysis observed. Prothrombin estimations carried out with human and rabbit brain, however, indicated equal thromboplastic activity.

From the results obtained in a few experiments it would appear that the specificity of the fibrinolytic enzyme produced by combination of the serum precursor with brain fibrinokinase is confined to the undenatured fibrin.

TABLE IV.—*Difference in Reactivity of Native and Heated Fibrin.*

Number of samples.	Fibrinolysis of Native fibrin. (1) In presence of serum.	(2) In absence of serum.	Fibrinolysis of heated fibrin. (3)
6 .	6	*Nil* .	*Nil*

For experiments in Column 3, Table IV, the fibrin deposited on glass rods was prepared as in previous experiments, but it was removed from its serum and immersed in boiling saline for 5 min. then allowed to cool and returned to the serum. The experimental results indicate quite clearly that the fibrinolysin was able to attack native fibrin only.

In further experiments attempts were made to concentrate the active brain factor. It could be shown that extraction of brain powder with distilled water and, in some cases, 0·4 per cent NaCl, did not produce active extracts, indicating that the principle is not of albumin nature.

Fractionation of saline extracts with ammonium sulphate was sometimes difficult to carry out because sediments would not settle even when centrifuged. In some instances, however, it was possible to obtain filterable precipitates at a concentration of between 35 per cent and 50 per cent ammonium sulphate saturation. Precipitates were dialysed against distilled water and the resulting suspension showed high fibrinolytic activity. The protein concentration of one such suspension was 25 mg. per cent protein N.

The active principle of the saline extract of brain could be adsorbed by alumina gel, but elution with phosphate buffer was unsuccessful.

Barium sulphate suspension did not adsorb the active principle from the brain preparation. Further, the active principle could be precipitated by acetone from the saline extract.

Experimental evidence brought forward is in favour of the assumption that brain fibrinokinase is saline soluble and present in the globulin fraction. Since several workers believe that there is no difference between the susceptibility of fibrinogen and fibrin to fibrinolysins, Christensen (1945), Tagnon, Davidson and Taylor (1942), it seemed of interest to investigate the reactions of a fibrinolysin produced by activation of the plasma precursor by streptokinase.

The potency of the preparation was adjusted to a fibrinolysis time between approximately 1 and 19 hours. Preliminary qualitative experiments indicated that fibrinolysin produced by streptokinase activation attacked fibrin preferentially to fibrinogen. Results for two different streptokinase concentrations are given in the following table.

TABLE V.—*Influence of Streptokinase on Fibrinolysis.*

Streptokinase concentration.	Fibrinolysis time in hours (1).	Fibrinogenolysis (2).
1	1	*Nil*
0·5	1–17	,,

The concentration of streptokinase is expressed in arbitrary units. Four plasma specimens were tested.

Results in Column 1, Table V, refer to experiments which were carried out in the presence of calcium ions, whilst those in Column 2 were carried out in the absence of calcium ions. They were added to this series when fibrin disappearance was noticed in the Column 1 series. The figures in the table refer to the time in hours until complete fibrin disappearance. The results indicate that fibrinogen was not destroyed in specimens incubated with streptokinase, although fibrin was attacked. Further, the streptokinase was not inactivated during the incubation period because after conversion of fibrinogen to fibrin, fibrinolysis occurred in 1 to 3 hours.

In order to investigate quantitatively the extent of fibrinolysis or fibrinogenolysis the following experiments were carried out.

Into three series of 50 ml. centrifuge tubes was introduced 1 ml. oxalated or citrated plasma (in the latter case specimens were obtained by adding 1 vol. 3·2 per cent sodium citrate to 9 vol. blood). The plasma was diluted with 22 ml. veronal buffer, pH 7·2, and in Series 1, 1 ml. 0·25 M calcium chloride was added. This was a control series.

Series 2 contained, in addition to the components of Series 1, 1 ml. of a suitably-diluted streptokinase preparation.

Series 3 contained diluted plasma and streptokinase but no calcium chloride. All the tubes were incubated at 37° C. When the fibrin gels in Series 2 disappeared completely, thrombin and calcium chloride was added to the tubes of Series 3, and the contents chilled in an ice bath. The tubes were spun as quickly as possible, and the deposited fibrin washed with isotonic saline until protein free. Protein estimations of the isolated fibrin were carried out by the biuret reaction. The results of these estimations are shown in Table VI.

The concentration of streptokinase used for the experiments shown in Table VI produced fibrinolysis in two specimens in 1·5 hours at 37° C. Two more specimens were treated simultaneously and they lysed in 45 min. Following addition of thrombin, clot formation occurred, but the quantitative collection

TABLE VI.—*Recovery of Fibrinogen Following Incubation of Human Plasma with Streptokinase.*

Series 1. Fibrin N, mg. per 100 ml.	Series 3. Fibrin N, mg. per 100 ml.
72	59
40	35

of the fibrin clots was not successful because losses of fibrin due to fibrinolysis occurred during the isolation procedure.

These results indicate that the fibrinolytic enzyme produced by streptokinase activation does attack fibrin in preference to fibrinogen.

DISCUSSION.

Normal body fluids containing the coagulation factors will clot under favourable conditions, but the dissolution of the clot is neither a common nor a physiological property. The exceptions are the dissolution of fibrin in the endometrium and the lytic action of prostatic fluid (Huggins and Neal, 1942). Human blood, after coagulation, ordinarily does not show fibrinolysis. But this process can be induced by a variety of factors *in vivo* as well as *in vitro*.

With regard to the derivation of the fibrinolysin, two possibilities have been suggested. According to one, the enzyme is derived from sources outside the blood, and Mole (1948) believes that endothelial lining of the vascular channels and body cavities are the source of the enzyme which under certain stimuli enters the circulation. Another theory assumes that normal human blood contains an inactive precursor of the enzyme which can be activated by a kinase to the active principle. This assumption has been supported by several independent observations. It originates from the findings of Tillet and Garner (1933) that fibrin clots prepared from human plasma undergo lysis when culture filtrates of certain strains of streptococci are added. It was shown by Milstone (1941) that human plasma contains a factor present in the euglobulin fraction which by combining with the streptococcal component produces fibrinolysis. Christensen (1945) and Christensen and McLeod (1945), from a study of the purified components, concluded that the lytic factor exists in plasma as zymogen and that it is activated by the streptococcal factor called streptokinase in a manner similar to the activation of trypsinogen by enterokinase. This concept has been adopted by Biggs, McFarlane and Pilling (1947) for fibrinolytic activity of human blood produced by physiological stimuli. With regard to specificity, it was observed by Tagnon *et al.* (1942) and by Christensen and McLeod (1945) that the enzyme is active against proteins other than fibrin and the latter authors therefore thought it advisable to abandon the term fibrinolysin and suggested plasmin instead. The experimental evidence brought forward in this paper is in agreement with the assumption that human plasma or serum contains the precursor and the kinase can be supplied from sources outside the blood; in this case, brain. The results show quite clearly that the enzyme appearing in the circulation following electrically-induced convulsions, or activation by brain fibrinokinase attacks fibrin without affecting fibrinogen. Mole (1948) has found that the enzyme present in post-mortem blood has similar properties. Further, fibrinolysin produced by activation of the plasma precursor by streptokinase under our experimental conditions showed also preferential attack of fibrin.

Christensen (1945) obtained equal breakdown rates with fibrin as well as fibrinogen. A possible explanation for the difference in results is that Christensen used fibrinogen isolated by salting out procedures whilst we used diluted plasma as source of fibrinogen. Because of the pronounced specificity the term profibrinolysin, as suggested by Loomis, George and Ryder (1947) for the precursor of the blood enzyme is to be preferred. Turning now to the source of the kinase the evidence submitted allows the conclusion that human adult brain contains this factor. It will be recalled that attempts by previous workers to extract the active tissue component have been unsuccessful (Fleisher and Loeb, 1915; Permin, 1947). Tagnon and Petermann (1949) found the activator in the microsome fraction of rat lungs. The ease with which brain yields the fibrinokinase in contrast to other organs is possibly due to the phospholipids which may have a solubilizing effect on fibrinokinase. In this connection it is interesting to record that Halse (1946) and Kaulla (1947) found the fibrinolysis due to parenteral administration of novocain, or even saline is correlated with an increase of plasmaphosphatides. That we are dealing with a kinase and not a proteolytic enzyme is evident from the fact that fibrin in the absence of serum was not altered by the brain extract. It is true that in some of the experiments the extracts were able to attack isolated fibrin, but this was always smaller than in the presence of serum and therefore cannot be explained solely by the presence of a proteolytic enzyme in brain. It has to be considered also that precipitated fibrin includes other material than fibrin and it is possible that it includes some profibrinolysin. The activation by brain fibrinokinase is species specific. The heat lability suggests that this principle is not identical with the thromboplastin of brain. The physicochemical properties would indicate that it is present in the globulin fraction.

SUMMARY.

Human adult brain contains a factor capable of converting profibrinolysin of human plasma into fibrinolysin. The brain fibrinokinase can be extracted by salt solutions and is present in the globulin fraction. The factor is species specific and not identical with thromboplastin. The enzyme produced by the combination of the plasma with the brain factor breaks down fibrin made by clotting procedures, but shows no action on plasma fibrinogen or heat-denatured fibrin.

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INVESTIGATIONS OF THE PROTEINS RELEASED BY SURVIVING TISSUE. ELECTROPHORETIC ANALYSIS OF PROTEIN RELEASED BY SURVIVING LIVER AND KIDNEY SLICES.

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In contrast to the increasing knowledge of the amino-acid composition of various purified proteins, little information has accumulated on the protein composition of different tissues. Fractionation of proteins by salting out methods was employed on extracts from tissues by Wolff (1905) and Schenck (1934). Luck (1936) obtained 4 protein components by extraction of liver with alcohol in the cold. Initiated by the work of Szent-Gyögyi, many studies of the muscle proteins have been made (reviewed by Pedersen, 1948). In connection with research on the site of the production of antibodies, immunological methods have been employed for the identification of proteins obtained from lymphocytes (White and Dougherty, 1944, 1945) or splenic tissue (Fagraeus, 1948). To the same end Roberts and White (1949) investigated by electrophoresis the proteins released into rat serum by homogenized rat liver. No recent report is available in which the protein compositions of different tissues from the same animal have been compared. The present experiments were undertaken to investigate the proteins which are released by surviving tissues.

EXPERIMENTAL.

Healthy albino rats of either sex, of between 200 and 300 g. body weight, were used. As much blood as possible (8 to 10 ml.) was withdrawn from the heart under deep ether anaesthesia before the animal was killed. Slices of liver and kidney were cut 0·2 to 0·3 mm. thick with a razor blade. The cortex only of the kidney was used.

In the later part of the experiment, liver, kidney and tumour slices from three rats which developed tumours on being fed 2-acetylaminofluorene were also studied. The primary growths were a mammary carcinoma, a pancreatic and a hepatic tumour. The kidneys of these rats were macroscopically normal; the livers, however, contained in all cases numerous small tumours of pinhead size, which could not be separated from the liver tissue. When slicing the tumour tissue care was taken to avoid necrotic material.

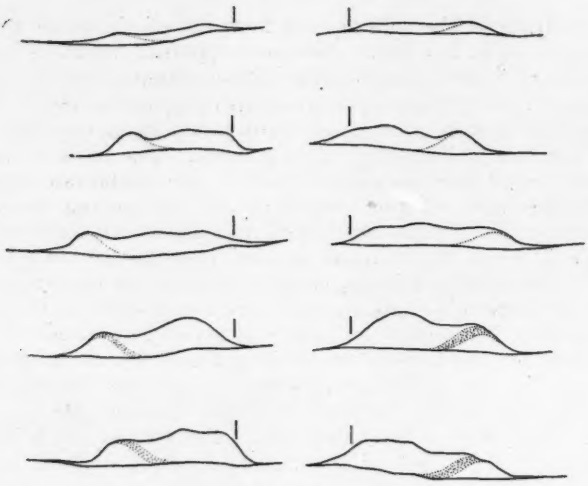
The slices were washed twice in sterile Tyrode solution in order to remove loose cells and serum, then placed in 2 ml. fresh sterile Tyrode solution and incubated in rotating tubes at 37° for 1½ to 2 hours. The supernatant fluid was poured off and centrifuged. The slices were drained between filter-paper, weighed, and dried at 110°. The supernatant fluid was diluted with buffer to

7·1 ml., and dialysed in the cold against 2000 ml. of phosphate buffer of pH 8 and ionic strength 0·1 for two days. The electrophoresis was made in the medium-size cell (10·5 ml.) of the Tiselius apparatus at a potential gradient of 5·2 V./cm. Under these conditions only about 1·6 watts are dissipated in the whole apparatus. The patterns were recorded photographically at the beginning and then at least twice during the run, which lasted up to 2 hours. The patterns were enlarged, traced, and the initial base-line superimposed. The mobilities were determined from the displacements of the ascending and descending boundaries. The electrode in the vessel which was closed was made alternatively positive or negative. The other electrode vessel was left open to the air. No corrections were applied to the mobilities for the volume change at the electrode (Longsworth, 1943), or for any other effect which may have contributed to the displacements of the boundaries. But, since the apparent mobilities seemed to depend consistently on whether the current was flowing towards or away from the closed side, the means for the mobilities in every group were calculated separately for each of the two current-directions and their average taken. The mobility of the fastest component only was measured. For comparison with the mobility of normal serum albumin sera of 7 normal rats were analysed. The migration velocities of the two albumin peaks were measured on the ascending patterns, reduced to zero protein concentration by means of the equation

$$V_1 = V_{al}/(1 + 0{\cdot}06\, C_i') \quad \text{(Hoch-Ligeti and Hoch, 1948)},$$

where V_1 = migration velocity at zero protein concentration, V_{al} = actual migration velocity, C_i' = apparent protein concentration, and 0·06 is a coefficient which had been determined experimentally for human serum albumin (Hoch, 1948), and the mobilities calculated by using the conductivity of the buffer. The migration velocities in all other experiments were not corrected to zero protein concentration since the correction would have been less than 1 per cent. The concentrations in the electrophoresis cell were calculated from the means of the total areas of the ascending and descending patterns. They ranged from 0·05 to 0·17 g./100 ml. Multiplication by the initial volume gave the total amount released by the tissue. This procedure was based on area measurements on patterns given by diluted rat serum at protein concentrations of 0·10 and 0·05 g./100 ml. The low concentration of protein in the extracts did not permit a resolution of the different globulin components, but the albumin peak was clearly distinguishable in the majority of the patterns. However, considerable uncertainty remained in the extrapolation of the albumin peak, the possible error range being from $\pm$ 10 to 30 per cent of the area under the albumin peak. The δ- and ε-boundaries were probably less than 1 per cent of the total area and accordingly not seen.

The relative proportions and the mobilities when measured at concentrations below 0·1 g./100 ml. might be subject to errors from causes not operative at the concentrations usually employed, such as heat convections which could shift the position of a boundary by " corrosion." Electrophoresis of a rat serum at protein concentrations of 1·2, 0·10 and 0·05 g./100 ml. (Fig. 1, No. 17 to 19) showed that under the conditions of the experiments heat convections did not seriously interfere, and that the apparent mean mobilities of the albumins were determined correctly (ascending 6·1 to 6·3, descending 5·8 to 6·0 10^{-5} cm.^{2} V.$^{-1}$



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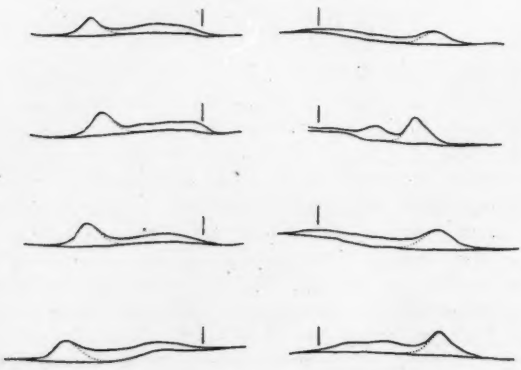

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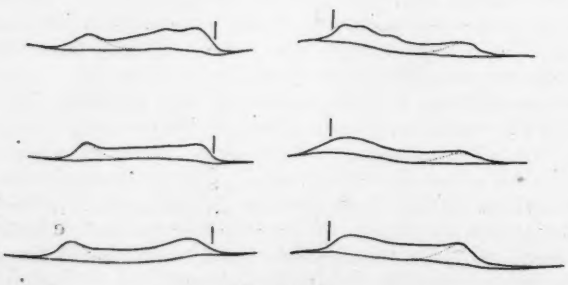

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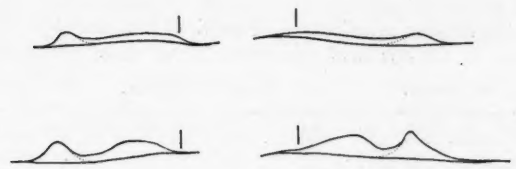

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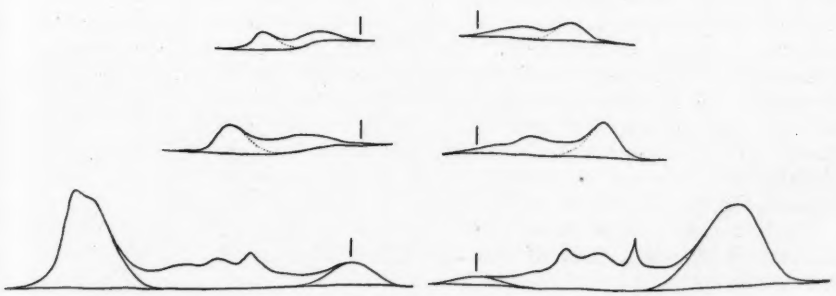

No. 17–19.

FIG. 1.—Tracings of electrophoretic patterns of proteins released by surviving tissue slices and of a serum electrolysed at low concentrations of protein. The figures in brackets designate the protein concentrations in the electrophoresis cell in g./100 ml.

1–5, normal livers (0·05–0·16).
6–9, normal kidneys (0·05–0·07).
10–12, livers of tumour-bearing rats (0·08–0·10).
13, 14, kidneys of tumour-bearing rats (0·05–0·08).
15, mammary tumour (0·05).
16, pancreatic tumour (0·17).
17–19, serum from rat which gave the patterns 10 and 16 at 1·2, 0·1 and 0·05 g. protein/100 ml.

sec.$^{-1}$). The apparent percentages of the albumin in the total were 58, 49 and 50 in the experiments at 1·2, 0·1 and 0·05 g./100 ml. respectively. Although the discrepancy between low and high concentrations was well within the uncertainty of placing the base line and dividing the areas, the values given in Table I for the fastest component in the tissue extracts may also tend to be too low.

TABLE I.—*Electrophoretic Analyses of Proteins Released by Surviving Tissue Slices and of Normal Rat Sera.*

		Normal rats.		Tumour-bearing rats.			Normal rats. Serum (7)*		
		Liver (5) mean (range).	Kidney (4) mean (r.).	Liver (3) mean (r.).	Kidney (2) mean (r.).	Tumour (2) mean (r.).	A_1 mean (r.).	A_2 mean (r.).	Mean mean (r.).
Mobility of fastest component	Asc.	5·9(±0·5)	5·9(±0·4)	6·2(±0·0)	6·1(±0·0)	6·1(±0·1)	6·05(±0·15)	5·4(±0·2)	5·7(±0·2)
	Desc.	6·1(±0·3)	5·8(±0·4)	6·2(±0·2)	6·2(±0·2)	6·0(±0·1)	..	..	..
Proportion of fastest component, % of total		29(25–38)	47(41–59)	25(±2)	38(±3)	52(±5)		66(63–71)	
Amount released, % of dry weight of slices		7·6(6·8–9·1)	6·4(4·8–8·8)	5·6(4·7–7·3)	6·2(±1·4)	6·0(±2·5)			

The bracketed figures in the headings designate the number of rats.
* Mobilities corrected to zero protein concentration. A = albumin.

RESULTS.

From the findings, which are summarized in Table I and Fig. 1, it can be seen that the surviving tissue released material the electrophoretic mobility of which ranged from that of the γ-globulin to that of the faster albumin component of normal rat serum.

In the following the components of the extracts will be designated by the terms used for the serum fractions, but by this no identity should be implied. The non-dialyzable non-protein material, which contributes to the area of the patterns, was not investigated. The albumin fraction in the extracts from normal liver and kidney was found to migrate with the same mobility as serum albumin. The mobility of this fraction was slightly greater with tumour-bearing animals, but the difference was not significant. The results of all experiments taken together appear to indicate that the mobility of a considerable proportion of the protein released by liver or kidney tissue was nearer to that of the faster albumin fraction of serum than to that of the slower fraction which in serum normally makes up the larger portion of the albumin. No component with a mobility faster than that of the albumin was detected. Nucleoproteins are more negatively charged than albumin, and they would migrate appreciably faster (van Winkle and France, 1948). It was of importance to ascertain whether nucleoprotein is released together with the other proteins, and Dr. E. R. Holiday and Dr. E. A. Johnson investigated the spectra of two extracts. These showed absorption in the regions of tyrosin, trytophane and phenylalanine, but there was no indication of an absorption maximum near 2600 Å. If nucleoprotein was present its concentration must have been less than 5 per cent of the total material extracted. The proportion of globulins + fibrinogen, particularly of γ-globulin + fibrinogen, was significantly higher in extracts from liver than from kidney or in normal serum. In all but one (No. 4) of the extracts from normal liver and liver of tumour-bearing rats the concentration of the γ-globulin was high. In two kidney extracts (Fig. 1, No. 7 and 14) the β-globulins were dominant and the γ-globulin + fibrinogen low. The protein released by the tumour tissues contained 47 and 57 per cent albumin. The amount of non-dialysable material released by the tissues varied from 3·5 to 9·1 per cent of the dry weight of the tissue after extraction, but there was no difference between the groups.

In order to assess to what extent blood which had not been washed out of the tissue could have contributed, the sediments of a liver and a kidney extract

from one animal (No. 4 and 9) were lysed and the oxyhaemoglobin measured in a colorimeter. Assuming 15 g. haemoglobin and 4 g. serum protein/100 ml. blood, the corresponding amounts of blood worked out to 0·007 and 0·005 ml. and the amounts of serum protein to 0·3 and 0·2 mg. Since the total amounts extracted were 11 and 5 mg., the contamination by serum proteins was therefore only 3 and 4 per cent for liver and kidney respectively.

The patterns of the serum, which was electrolysed also at concentrations as low as those used with the tissue extracts, were compared with those given by liver and tumour tissue slices of the same rat (Fig. 1, No. 10, 16 and 17 to 19). The slowest peak in the pattern of the liver proteins (No. 10) had the mobility of the γ-globulin of serum, and it made up an appreciable proportion of the total area. In the tumour extract (No. 16) the largest peak, apart from the albumin, migrated with the mobility of fibrinogen. The pattern of the serum (No. 19) deviated from the normal (Hoch-Ligeti, Hoch and Goodall, 1949) in several respects. The larger portion of the albumin peak was made up by the faster component. The proportion of the α-globulin was high, there were two β-globulin peaks, and the proportion of the γ-globulin was low. In the patterns obtained at low protein concentration the globulin components were not resolved. The serum patterns differed from either of the patterns of the proteins released by liver or by tumour tissue.

DISCUSSION.

In order to be able to investigate the protein released by surviving, well-oxygenated slices of relatively small organs, it was necessary to conduct the electrophoresis experiments at unusally low protein concentrations. The advantage of obtaining information from small quantities of material was considered to outweigh the difficulties encountered in resolving the components.

No protein was released which had a mobility outside the range found for the proteins of normal serum. It was of importance to ascertain that not all the protein released by the tissue originated from the blood that was left included in the slices of exsanguinated rats even after repeated washings with Tyrode solution. Apart from the experimental finding that the percentage of blood in the tissue extract, as calculated from the content of red cells, was low, the fact that liver and kidney slices gave consistently different types of patterns excludes this possibility.

The high percentage of albumin released by the kidney may be of importance in connection with the origin of the albumin excreted in nephritis.

The electrophoretic patterns of the proteins released by the liver and the kidney of rats bearing tumours induced by feeding 2-acetylaminofluorene did not differ from those of the proteins released by these tissues of healthy rats, although the liver slices included visible neoplastic material. The tumour tissues, however, released a high percentage of albumin, the significance of which is difficult to assess before homologous normal tissue has been investigated. Here also no component with a mobility greater than that of the albumin was found.

It is remarkable that the amounts of material migrating with the mobility of α-globulin was small, in view of the work of Roberts and White (1949), who found the main increase, after incubation of homogenized liver in serum, to occur in the region of the α-globulin.

SUMMARY.

The proteins released by surviving slices of livers and kidneys of rats were analysed by electrophoresis.

No non-dialysable material was released the mobility of which was outside the range found for the proteins of normal rat serum.

The proportions of the fastest component in the total were determined. The proportion of this component was 25 per cent in the material released by liver slices, and about 50 per cent in the material released by kidney slices.

No difference could be found between the patterns from liver or kidney tissues of healthy and of tumour-bearing rats. Tumour tissue itself released a high percentage of the component which migrated with the mobility of albumin.

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RESTORATION OF THE ABILITY OF SOME HEATED ANTISERA TO FLOCCULATE SPECIFICALLY THEIR ANTIGENS.

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WHEN mixtures of different proteins are being denatured by heat their particles may combine to form complex aggregates. An antigen in such complex may fail to be precipitated by its antibodies, although it still combines with them and inhibits the precipitation of unheated antigen (Kleczkowski 1945*b*, 1949). The ability of some antigens to be precipitated can be restored by incubating the complex with pepsin (Kleczkowski, 1945*a*).

Antibodies undergoing initial stages of heat denaturation can also form complexes with other proteins and subsequently fail to flocculate (i.e. agglutinate or precipitate) their antigens, although they may still be able to combine with them and to inhibit their flocculation by untreated antisera (Kleczkowski, 1941, 1945*b*). The experiments described below show that antibodies in such complexes can also be converted by incubation with a proteolytic enzyme into a form in which they again flocculate their antigens.

MATERIALS AND METHODS.

The antigens used were tobacco mosaic virus, tomato bushy stunt virus, horse serum albumin and a strain of clover nodule bacteria.

Virus preparations.—Solutions of purified viruses were prepared as described by Bawden and Pirie (1943).

Bacterial suspension.—Cultures of a strain of pea nodule bacteria ("Orkney I"), grown on agar slopes, were suspended in 0·9 per cent NaCl. Floccules of slime were removed by filtration through cotton wool.

Horse and rabbit serum albumins.—Globulins were precipitated by half saturation of the sera with ammonium sulphate and removed by filtration. Albumins were precipitated from the filtrates by 2/3 saturation with ammonium sulphate. The precipitates were dissolved in, and dialysed against H_2O. A few pieces of thymol were placed in the dialysing sacks as an antiseptic.

"Insoluble euglobulin" of rabbit serum.—The precipitate obtained from rabbit serum by one-third saturation with ammonium sulphate was separated by centrifugation, dissolved in, and dialysed against H_2O. The precipitate formed during dialysis was centrifuged down, washed in H_2O and dissolved in 0·9 per cent NaCl.

Antisera were prepared by intravenous injections of rabbits. 4 to 8 mg. of the viruses, 10 to 40 mg. of horse serum albumin and 10^{10} bacteria were injected into different rabbits twice a week for three weeks. The animals were bled 8 to 10 days after the last injection.

Euglobulin and whole globulin fractions of antisera were prepared by precipitation with 1/3 and 1/2 saturation with ammonium sulphate, respectively. The precipitates were dissolved in H_2O and dialysed against 0·9 per cent NaCl.

Trypsin.—A commercial preparation of trypsin obtained from the British Drug Houses Ltd. was used in most experiments. Suspensions of the stated concentration were made in buffer solutions, and undissolved residue was removed by centrifugation.

In a few experiments crystallized chymotrypsin was used. This was prepared as described by Northrop (1938).

Serological precipitin tests were made by adding 1 ml. of varying dilutions of antiserum preparations to a series of tubes, each containing 1 ml. of antigen solution at a constant concentration or *vice-versa*. The tubes were placed in a water-bath at 50° C. with columns of fluid half immersed so that mixing occurred by convection.

To test for inhibition of flocculation by variously treated antiserum preparations, 0·1 ml. of suitably diluted untreated antiserum was added to the tubes in which there was no flocculation after 3 hours' incubation. Continued absence of flocculation was taken as evidence of inhibition.

With the viruses and the bacteria, antigen was used at a constant concentration. The viruses were used as 0·005 per cent solutions and the bacteria as suspensions containing about 10^9 organisms per 1 ml. Bacterial suspensions were heated before use for 10 minutes at 80° to destroy " H " type agglutinins, so that agglutination was exclusively " O " type.

In experiments with horse serum albumin, antiserum concentration was kept constant, and antigen concentrations varied.

Nitrogen estimations were made by the micro-Kjeldahl method and the amounts of nitrogen were translated into protein by multiplication by 6·4.

May and Rose's colorimetric test for tryptophan was used in a modification previously described (Kleczkowski, 1949).

RESULTS.

Restoration of Flocculating Power of Preparations Containing Inhibiting Antibody Complexes.

Tables I and II give the results of experiments to test the effect of B.D.H trypsin at pH values around 7 on the behaviour of unheated and heated antisera and of unheated and heated euglobulin fractions of the antisera. The euglobulin fractions were heated in the presence and absence of rabbit serum albumin. Similar results were obtained when 0·05 per cent crystallized chymotrypsin was substituted for 0·2 per cent B.D.H. trypsin.

As a result of heating all the antisera ceased to flocculate and inhibited, and so, too, did their euglobulin fractions heated in the presence of albumin. Incubation with trypsin did not affect the behaviour of any antiserum preparations unless they had previously been heated. It restored the flocculating power of heated antisera to tobacco mosaic virus, bushy stunt virus and to the bacteria, although the treated antisera caused flocculation more slowly and gave lower titres than the original antisera. By contrast, trypsin destroyed the inhibiting power of heated antiserum to horse serum albumin without restoring its flocculating power. The behaviour of the euglobulin fractions heated in the presence of

albumin was affected by incubation with trypsin similarly to that of heated whole antisera.

Results obtained with different antisera to tobacco mosaic virus varied. The flocculating power of some of them was reduced but not destroyed by heating in conditions similar to those specified in Table I. The virus was flocculated more slowly by such heated antisera, which usually gave a zone of antibody-excess inhibition not shown by the original antiserum. After incubating the heated antisera with trypsin, the speed of flocculation was increased, although not to that of the original antiserum, and the zone of inhibition disappeared.

TABLE I.—*The Effects of Trypsin on the Behaviour of Heated and Unheated Antisera.*

Mixtures incubated for 24 hours at 37°.			Precipitin test. Further dilutions of antisera. 1/								
2·5 ml. of antiserum dil. 1/10.	Buffer (ml.).	Trypsin (ml.).	1.	2.	4.	8.	16.	32.	64.	128.	256.
Antiserum to Tobacco Mosaic Virus.											
Unheated	0·5	..	+++	+++	+++	+++	++	+	−	..	..
,,	..	0·5	+++	+++	+++	+++	++	+	−	..	..
Heated	0·5	..	i	i	i	o	o	o	o	..	..
,,	..	0·5	+++	+++	+++	++	+	−	−	..	..
Antiserum to Bushy Stunt Virus.											
Unheated	0·5	..	+++	+++	+++	+++	++	+	−	..	..
,,	..	0·5	+++	+++	+++	+++	++	+	−	..	..
Heated	0·5	..	i	i	i	i	i	i	o	..	..
,,	..	0·5	+++	+++	+++	++	+	−	−	..	..
Antiserum to the Bacteria.											
Unheated	0·5	..	..	+++	+++	+++	+++	+++	++	+	−
,,	..	0·5	..	+++	+++	+++	+++	+++	++	+	−
Heated	0·5	..	..	i	i	i	i	i	o	o	o
,,	..	0·5	..	+++	+++	+++	++	+	−	−	−

			Antiserum was further diluted so that the final dilution was 1/20. Mg. of antigen × 100.								
			100.	50.	25.	12·5.	6·25.	3·1.	1·6.	0.8.	0·4.
Antiserum to Horse Serum Albumin.											
Unheated	0·5	..	−	++	+++	+++	+++	++	+	±	−
,,	..	0·5	−	++	+++	+++	+++	++	+	±	−
Heated	0·5	..	i	i	i	i	i	i	i	i	..
,,	..	0·5	o	o	o	o	o	o	o	o	..

Antisera were heated for 10 minutes at 75° at a dilution of 1/10 in 0·9 per cent NaCl.

Tests for inhibition were made by adding 0·1 ml. of virus antisera at a dilution of 1/5, of bacterial antiserum at 1/20 and of horse albumin antiserum at 1/2.

Buffer: 0·2 M boric acid—borax buffer at pH 7·4.

Trypsin: 0·2 per cent solution of B.D.H. trypsin in the buffer.

\+ signs indicate the presence and the degree of flocculation.

− signs indicate the absence of flocculation if the test for inhibition was not made.

i signs indicate the presence of inhibition.

o signs indicate the absence of inhibition.

Table II shows that all the euglobulin fractions of antisera heated alone still flocculated their antigens. Subsequent incubation with trypsin did not destroy the flocculating power of the euglobulins from the antisera to the viruses, although flocculation was delayed and the titre slightly reduced. Both speed and titre

TABLE II.—*The Effects of Trypsin on the Behaviour of Heated and Unheated Euglobulin Fractions of Antisera.*

E = euglobulin solutions at a concentration of 0·1 per cent.
E + A = mixtures of euglobulin and rabbit serum albumin containing 0·1 per cent of euglobulin and 0·5 per cent albumin.
All the solutions contained 0·9 per cent NaCl and the pH was about 6·8.
The solutions were heated for 10 minutes at 75°.

Mixtures incubated for 24 hours at 37°.			Precipitin test. Dilutions of incubated preparations. 1/						
2·5 ml. of—	Buffer (ml.)	Trypsin (ml.)	1.	2.	4.	8.	16.	32.	64. (ml.).
Euglobulin of Antiserum to Tobacco Mosaic Virus.									
E Unheated	0·5	..	+++	+++	++	+	−	−	−
E „	..	0·5	+++	+++	++	+	−	−	−
E heated	0·5	..	+++	+++	+++	+	−	−	−
E „	..	0·5	+++	++	+	−	−	−	−
E + A heated	0·5	..	i	i	o	o	o	o	o
E + A „	..	0·5	+++	++	+	−	−	−	−
Euglobulin of Antiserum to Bushy Stunt Virus.									
E unheated	0·5	..	+++	+++	+++	+++	++	+	−
E „	..	0·5	+++	+++	+++	+++	++	+	−
E heated	0·5	..	+	++	+++	+++	++	+	−
E „	..	0·5	++	+++	+++	+++	+	−	−
E+A heated	0·5	..	i	i	i	i	i	i	o
E+A „	..	0·5	++	+++	+++	+++	+	−	−

Euglobulin of Antiserum to Horse Serum Albumin.
Incubated preparations were used undiluted.

			Mg. of antigen × 100.							
			100.	50.	25.	12·5.	6·25.	3·1	1·6.	0·8.	0·4.
E unheated	0·5	..	−	+	+++	+++	+++	+++	++	+	±
E „	..	0·5	−	+	+++	+++	+++	+++	++	+	±
E heated	0·5	..	++	+++	+++	+++	+++	++	+	±	−
E „	..	0·5	o	o	o	o	o	o	o	o	..
E+A heated	0·5	..	i	i	i	i	i	i	i	i	..
E+A „	..	0·5	o	o	o	o	o	o	o	o	..

Buffer and trypsin solutions, method of testing for inhibition and symbols used here are the same as those shown in Table I.

of flocculation resembled those given by trypsin-treated euglobulins that had been heated in the presence of albumin. By contrast, incubation with trypsin destroyed the flocculating power of heated euglobulin from the antiserum to horse serum albumin. No detectable inhibiting power was developed.

The behaviour of heated euglobulin fractions depended on whether or not the albumin was present during heating. After the heated fractions had been incubated with trypsin, their behaviour was no longer influenced by the presence or absence of the albumin. It is obvious, therefore, that the non-flocculating and inhibiting complexes formed during heating between partially denatured molecules of antibodies and those of the albumin were changed by trypsin, and the experiments described in the following sections were made to obtain some facts that would help in interpreting the change.

At first sight it seems as though heated antibodies to horse serum albumin differ from those to the other three antigens, for they appear to be destroyed by

trypsin, whereas the other three are not. There is, however, some evidence that this is not so. Although trypsin-treated heated antibodies to horse serum albumin neither precipitated horse serum albumin nor inhibited its precipitation by unchanged antiserum, they did affect the reaction between horse serum albumin and unchanged antiserum. Precipitation occurred in the tube that contained 1 mg. of the antigen, an amount that would normally have caused antigen-excess inhibition. This was not because some horse serum albumin was hydrolysed by the trypsin present in the heated antiserum, because adding the same amount of trypsin to unheated antiserum did not affect its behaviour. The phenomenon suggested that antibodies, first altered by heat and then by trypsin, can still combine with horse serum albumin, but that the combination does not lead to precipitation or inhibit precipitation by unaltered antibodies. This interpretation was supported by the results of quantitative tests described below.

A Quantitative Estimation of Disaggregation by Trypsin of Euglobulin-Albumin Complex.

Table III shows the results of a quantitative test of the disaggregating effect of trypsin on a heat-produced complex between "insoluble euglobulin" and albumin.

Three solutions containing

A. 0·2 per cent of "insoluble euglobulin,"

B. 0·2 per cent of "insoluble euglobulin" and 1 per cent of rabbit serum albumin,

C. 1 per cent of rabbit serum albumin,

all in M/15 phosphate buffer at pH 6·8, were heated for 10 minutes at 80° C., when they were cooled and two samples of 10 ml. were taken from each. Two ml. of the M/15 phosphate buffer were added to one sample and 2 ml. of a 0·2 per cent solution of B.D.H. trypsin in the buffer were added to the other. The mixtures were incubated for 3 hours at 37° C., when 6 ml. of a saturated solution of ammonium sulphate were added to each. No precipitate was formed in the solutions containing the albumin heated alone (Sol. C, Tests No. 5 and 6). The precipi-

TABLE III.—*Precipitating Proteins by Ammonium Sulphate from Variously Treated Solutions Containing Euglobulin and/or Albumin.*

Test number.	The composition of heated mixtures. 0·4% euglobulin (ml.).	2% albumin (ml.).	Buffer (ml.).	Added after heating. Buffer (ml.).	0·2% trypsin (ml.)	Precipitate obtained by adding 6 ml. of a saturated solution of $(NH_4)_2SO_4$. Protein (mg.).	Equivalent* euglobulin (mg.).	Approximate composition of the precipitate. Euglobulin (mg.).	Albumin (mg.).
1	5	..	5	2	..	18·0	..	18·0	..
2	5	..	5	..	2	7·5	7·8	7·5	..
3	5	5	..	2	..	42·0	22·5	15·0	27·0
4	5	5	..	..	2	18·5	15·0	13·5	5·0
5	..	5	5	2	..	0	..	..	..
6	..	5	5	..	2	0	..	..	..

The solutions were incubated for 3 hours at 37°.

* i.e. The amount of euglobulin that, if suspended in the same volume of fluid as the precipitated protein, would give the same intensity of colour in May and Rose's test.

tates formed in all the other solutions were sedimented by centrifugation for 10 minutes at 8000 r.p.m., washed three times an a 5 per cent solution of trichloroacetic acid, and used to estimate the total protein (micro-Kjeldahl) and the relative colour intensity given in the modified May and Rose's test for tryptophan.

Incubation with trypsin rendered 60 per cent of the euglobulin heated alone unprecipitable by 1/3 saturated ammonium sulphate (Test No. 2). The part that remained precipitable gave the same intensity of colour as an equal amount of the original euglobulin.

More precipitate was obtained with 1/3 saturated ammonium sulphate from the heated euglobulin-albumin mixture (Test No. 3) than the total amount of euglobulin present. The precipitate therefore contained albumin, which was probably combined into a complex with euglobulin, for albumin heated alone was not precipitable by 1/3 saturated ammonium sulphate (Test No. 5). To find the amounts of euglobulin and albumin in the precipitate, use was made of the fact that euglobulin gave a colour intensity equal to that given by albumin when about 3·7 times more concentrated. Thus the amount of euglobulin (x) in the presipitate in Test No. 3 could be computed according to the equation—

$$z = x + (m - x)/3{\cdot}7$$

from the total amount of protein (m) and from the equivalent amount of euglobulin (z) that, when suspended in the same volume of water as the precipitate, gave the same intensity of colour. About 35 per cent of the precipitate was euglobulin, and over 80 per cent of the total euglobulin in the heated mixture was in the precipitate. By contrast, after the heated euglobulin-albumin mixture was incubated with trypsin, about 70 per cent of the precipitate produced by 1/3 saturation with ammonium sulphate consisted of euglobulin, and about 75 per cent of the total euglobulin present in the heated mixture was in the precipitate (Test No. 4). Thus trypsin descreased the amount of albumin combined with euglobulin, at least in the complex still precipitable by 1/3 saturated ammonium sulphate, and so presumably had disaggregated the complex.

Activity of Antibodies that have been Altered by Heat.

Two alternative explanations can be offered for the recovery of flocculating power when heated antisera are incubated with trypsin. First, the trypsin may disaggregate complexes formed between antibodies and other proteins and release changed antibodies in the form in which they are able to flocculate their antigens. Secondly, the complexes may be wholly destroyed by trypsin, their inhibiting effect thus removed, and the restored flocculating power may be entirely due to the action of antibodies that remained unaltered by heat. Experiments were made to see whether or not heat-changed antibodies treated with trypsin can flocculate their antigens, by separating heat-changed antibodies from any that may have remained unaltered by heat. This was done by making use of the fact that, whereas unheated serum proteins do not form a pellet when centrifuged for 1/2 hour at 40,000 r.p.m., heated serum proteins do because of aggregation following denaturation. It is assumed that during sedimentation they do not carry with them any significant quantity of unaltered proteins present in the solution.

Table IV shows the results of an experiment in which heated (10 min. at 80° C.)

TABLE IV.—*Sedimentation of Heat-denatured Antibodies to Tobacco Mosaic Virus by High-speed Centrifugation.*

0·1 per cent solution of whole globulin from antiserum to the virus was used. It contained 0·9 per cent NaCl and the pH was adjusted to about 6·8.

The mixtures incubated 4 hours at room temperature. 2·5 ml. of—		Buffer (ml.).	0·2% trypsin (ml.)	Precipitin test. Dilutions of the preparations. 1/ 1.	2.	4.	8.	16.	32.
Globulin unheated, not centrifuged		0·5	..	+++	+++	+++	++	+	−
„ heated not centrifuged		0·5	..	+++	+	−	−	−	−
„ „ „ „		..	0·5	+++	++	+	−	−	−
„ unheated, centrifuged*		0·5	..	+++	+++	+++	++	+	−
„ heated and centrifuged	Supernatant fluid	0·5	..	+	−	−	−	−	−
	„ „	..	0·5	+	−	−	−	−	−
	Resuspended pellet†	0·5	..	+	−	−	−	−	−
	„ „	..	0·5	++	+	±	−	−	−

* There was no pellet.

† The material was not completely dissolved. The undissolved part was removed by centrifugation for 5 minutes at 8000 r.p.m.

and unheated whole globulin from an antiserum to tobacco mosaic virus, were centrifuged for 1/2 hour at 40,000 r.p.m. No pellet was sedimented from the unheated solution. The pellet from the heated solution was suspended in a volume of 0·9 per cent NaCl equal to the volume of the fluid from which it came. Two samples were taken from both the supernatant fluid and from the resuspended pellet. The samples were incubated for 4 hours at room temperature, one from each with 0·2 M boric acid-borax buffer at pH 7·4, and the other with B.D.H. trypsin dissolved in the buffer. The pellet dissolved completely after incubation with trypsin, but only partly after incubation without trypsin. The undissolved material was removed by low-speed centrifugation and the supernatant fluid was used for the precipitin test.

The unheated antibodies were not sedimented by centrifugation, whereas about 75 per cent of antibodies in the heated solution were, and they precipitated their antigen after they had been resuspended and dissolved with the aid of trypsin.

In another experiment a solution containing 0·1 per cent of whole globulin from antiserum to the bacteria, 0·5 per cent of rabbit serum albumin and 0·8 per cent NaCl, was heated for 10 minutes at 80° C. at pH 6·8 and then centrifuged for 1/2 hour at 40,000 r.p.m. The redissolved pellet did not agglutinate the bacteria and inhibited their agglutination by unheated antiserum. After 3 hours' incubation with trypsin at pH about 7·0, the redissolved pellet agglutinated the bacteria.

Bacterial agglutinins can be coagulated by heat and then brought into solution by incubation with trypsin. A solution containing 0·2 per cent of whole globulin from the bacterial antiserum and 0·9 per cent NaCl was heated for 10 minutes at 77·5° C. and pH 6·0. The coagulum that was formed was sedimented by centrifugation at 8000 r.p.m., suspended in 0·9 per cent NaCl solution and dissolved by incubation with trypsin for 3 hours at 37° and pH 7·0; the resulting solution agglutinated the bacteria. This agrees with Pope's (1938, 1939) demonstration that antitoxin can be recovered from heat-coagulated protein of diphtherial

TABLE V.—*Effect of Trypsin on Unheated and Heated Antiserum to Horse Serum Albumin.*

Test number.	Antiserum.	Horse serum albumin (mg.). 0·31.	0·45.	0·63.	0·9.	1·25.	2·5.	
1	0·5 ml. of unheated antiserum no trypsin added	..	3·0	3·1	3·2	2·9	2·3	mg. precipitate.
		..	−	+	++	+++	+++	antigen excess.
		..	++	+	−	−	−	antibody excess.
2	0·5 ml. of unheated antiserum trypsin added immediately before test was made	..	..	3·0	..	2·9	2·4	mg. precipitate.
		..	..	+	..	+++	+++	antigen excess.
		..	..	+	..	−	−	antibody excess.
3	0·5 ml. of unheated antiserum incubated with trypsin	2·45	..	3·0	..	2·6	2·2	mg. precipitate.
		−	..	+	..	+++	+++	antigen excess.
		+++	..	+	..	−	−	antibody excess.
4	0·5 ml. of heated antiserum incubated with trypsin + 0·5 ml. of unheated antiserum	..	3·5	..	4·2	..	..	mg. precipitate
		..	−	..	++	..	..	antigen excess.
		..	++	..	+	..	..	antibody excess.
5	0·5 ml. of heated heterologous antiserum incubated with trypsin + 0·5 ml. of unheated antiserum	..	2·9	..	3·0	..	..	mg. precipitate.
		..	−	..	++	..	..	antigen excess.
		..	++	..	−	..	..	antibody excess.

antisera by treatment with different proteolytic enzymes, and the antibodies so recovered have antitoxic activity *in vivo* and flocculate the toxin *in vitro*. The release by a proteolytic enzyme of active antibodies from a heat-produced coagulum would seem to be basically the same phenomenon as the release of active antibodies from heat-produced soluble complexes formed between antibodies and other serum proteins as a result of initial stages of denaturation.

A Quantitative Demonstration of the Ability of Trypsin-treated Heated Antibodies to Horse Serum Albumin to Combine with their Antigen

An experiment was made to see whether heated antibodies to horse serum albumin are destroyed by incubation with trypsin at pH values around 7, or whether they are only altered so that they still combine with the antigen, but fail to cause precipitation. If they combine without causing inhibition, they should increase the amount of the precipitate formed by unheated antibodies. To test this possibility it was also necessary to find whether trypsin affects the amount of precipitate formed by combination between horse albumin and unheated antiserum.

In all the tests 4 ml. was the total volume of each antigen-antibody mixture, 0·5 ml. of 0·9 per cent NaCl solution containing different amounts of antigen, was mixed with 3·5 ml. of various antiserum preparations, both cooled to 0° C. before mixing. The mixtures were stirred and left for 24 hours in an ice-bath. The precipitates were centrifuged, washed with cold 0·9 per cent. NaCl and the protein estimated by the micro-Kjeldahl method.

In test No. 1 (Table V) 0·5 ml. of unheated antiserum was mixed with 2·5 ml. of 0·9 per cent NaCl and with 0·5 ml. of 0·2 M boric acid-borax buffer at pH 7·4. In all the others the 0·5 ml. of the buffer was also an 0·2 per cent solution of B.D.H. trypsin. In Test No. 2, trypsin solution was added immediately before adding the antigen, whereas in Test No. 3 the antiserum was incubated with trypsin for 48 hours at 37° C. In Tests No. 4 and 5 the antiserum and a heterologous

antiserum to tobacco mosaic virus were diluted 1/5 with 0·9 per cent NaCl, heated for 10 minutes at 75° C., when a volume of the trypsin solution equal to the volume of undiluted antiserum was added and the mixtures were incubated for 48 hours at 37° C. They were first mixed with solutions of the antigen and unheated undiluted antiserum was added after 1 hour's incubation at 0° C.

When trypsin was added to unheated antiserum and then incubated at 37° C. before adding the antigen, the amount of the precipitates was slightly reduced (cf. Tests No. 1 and 3); without incubation the trypsin had no effect (Test No. 2).

Adding heated antiserum that had been incubated with trypsin, increased the amount of precipitate formed when unheated antiserum reacted with the antigen (Test No. 4), whereas adding a similarly treated heterologous antiserum did not (Test No. 5). Antibodies present in the heated antiserum that had been incubated with trypsin, therefore, retained their ability to combine with horse serum albumin and were precipitated together with it by unchanged antibodies.

DISCUSSION.

There are numerous records of altering antibodies by various proteolytic enzymes without destroying their specific activity. The results obtained by estimation of altered ratios of antibody to antigen in specific precipitates (Pappenheimer and Robinson, 1937; Pope, 1938; Grabar, 1938) or by measuring sedimentation constants of antibody preparations treated with proteolytic enzymes (Petermann and Pappenheimer, 1941; Rothen, 1941–42; Petermann, 1942), all point to the conclusion that some antibodies can retain their specific functions after their molecules have been split to about half of their original size. Apparently only one of the two parts into which the original molecule has been split carries antibody activity, and there are also indications that this part is more resistant to further hydrolysis by the proteolytic enzyme used and less coagulable by heat. When trypsin is used, a pretreatment such as exposure to heat or to acid, seems to be essential (Pope, 1939; Northrop, 1941–42).

All the heated antibodies used in this work may have been altered by trypsin, their molecules having been reduced in size. In spite of the alteration the antibodies against all the four antigens retained their ability to combine specifically. Heated antibodies to horse serum albumin may have been altered by trypsin differently from heated antibodies against the other three antigens, but this assumption does not seem necessary. All four could have been affected similarly, and the different behaviour of the compounds formed between them and their antigens could reflect differences in some properties of the antigens, such as different solubilities.

SUMMARY.

The ability of some antisera to flocculate, i.e. precipitate or agglutinate, their antigens, which is lost by heating, can be restored by incubation with trypsin. The restoration occurs because complexes, formed between antibodies and other serum proteins during the initial stages of heat denaturation, are disaggregated. Trypsin also causes some changes in the molecules of heat-denatured antibodies, probably reducing their size. The changed antibodies still combine specifically with their antigens, but whether or not the combination leads to flocculation depends on the character of the antigen. Tobacco mosaic virus, tomato bushy

stunt virus and a strain of clover nodule bacteria were flocculated by trypsin-treated, heat-denatured antibodies, but horse serum albumin was not.

I thank Mr. N. W. Pirie, F.R.S., for doing all the high-speed centrifugations.

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AN EASY METHOD FOR COUNTING BACTERIAL COLONIES IN ROLL TUBES.

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JULIUS (1938) described an ingenious method for determining viable counts in roll tubes. He inoculated small volumes (3 ml.) of cooled molten nutrient agar in uniform insulin bottles with drops of inoculum delivered from calibrated micro-pipettes. The inoculated bottles were then spun in a horizontal position until the agar had set. After incubation the colonies in the bottles were counted with the aid of a mechanical device in which the bottles were slowly rotated and moved in the direction of their long axes past a slit through which the colonies were observed one by one. In this way the danger of counting a given colony more than once was practically eliminated.

In the present modification more or less the same procedures are followed, except that another method of colony counting is used. One ml. amounts of nutrient agar at 43 to 45° C. in uniform 10 ml. insulin bottles (1·75 cm. internal diameter, 3·8 cm. height up to shoulder) are inoculated from calibrated Pasteur pipettes (tips of uniform external diameter) with suitable dilutions of the inoculum. After inoculation the bottles are rotated in an electric spinner (Fig. 1) at 500 r.p.m. for one minute, by which time the agar sets in a uniform film of about 0·5 mm. thickness. The bottles are left in a horizontal position for a further 15 minutes and then incubated upside down.

The colonies are counted by projecting enlarged images of known sections of the bottles on to a screen or a wall by means of an epidiascope. The bottles are closely fitted into a brass cylinder (Fig. 2) lying horizontally between the mirror and the light source of the epidiascope. The brass cylinder has two longitudinal slits opposite each other in the path of the light; the upper slit is of known dimensions (3·5 cm. × 0·2 cm.), the lower slit is slightly longer and wider. The epidiascope is so focused that the colonies directly under the upper slit are in focus, and those directly above the lower slit are invisible. By this means a projected image of the upper slit and the colonies in it can be obtained with a ten-fold magnification and the colonies can easily be counted (Fig. 3). All the whole colonies in the slit area, and those parts of colonies appearing on two adjacent edges of the slit are counted. The number of slit areas equivalent to the inoculated area is given by the expression $\pi\ dh/wl$, where d = internal diameter of bottle, h = internal height of bottle up to the shoulder, w = width of slit, l = length of slit (30 slit areas are equivalent to one of our bottles). The bottle is rotated about 20 degrees at a time inside the cylinder and the colonies appearing in 20 slit areas are counted.

Surface and sub-surface colonies are counted. With *Bact. coli* growing on heart agar the ratio of the number of sub-surface to surface colonies increases with increasing total count. On the average the diameter of surface colonies is about 7 times as great as that of sub-surface colonies.

When 12 bottles were inoculated with equal volumes of a suspension of *Bact. coli* and the colonies appearing in 20 slits for each bottle were counted, the following results were obtained :

The smallest number of colonies observed in a slit area was 20, the largest was 52. The average slit count (20 slits) per bottle varied from 32·7 to 37·4 ; the mean value for 12 bottles was 34·6, with a standard deviation of $\pm$ 1·1 per cent. When the mean number of colonies per slit area was 13·2 the standard deviation was $\pm$ 2·3 per cent. Taking in each case the 3 bottles with values furthest from the mean the standard deviations were $\pm$ 4·4 per cent and $\pm$ 7·0 per cent. respectively.

This note deals only with a method for counting colonies in roll tubes and with the sampling errors involved ; we are not concerned with the relative merits of the roll tube and the plate methods of determining viable counts. It might be thought, however, that a concentration of about 35 colonies per slit area, about 1000 colonies per bottle, would give rise to errors due to overcrowding. Table I shows the results of a single experiment in which 4 drops of the dilutions of inoculum shown were inoculated into the agar in bottles and spread on the surface of agar in plates. The figures are the means obtained for 4 bottles or plates per dilution.

TABLE I.

Dilution of inoculum.	Colonies per bottle.	Colonies per plate.
1/1 million	1776	Not countable*
1/10 million	198†	188
1/100 million	19†	17·2

* Much confluent growth.
† Slit not used—all the colonies in the bottle counted.

Practically no confluent growth was observed in bottles containing as many as 2000 colonies.

We are grateful to Dr. A. G. Sanders for suggesting the use of an epidiascope in the first instance as a means of measuring the size of colonies.

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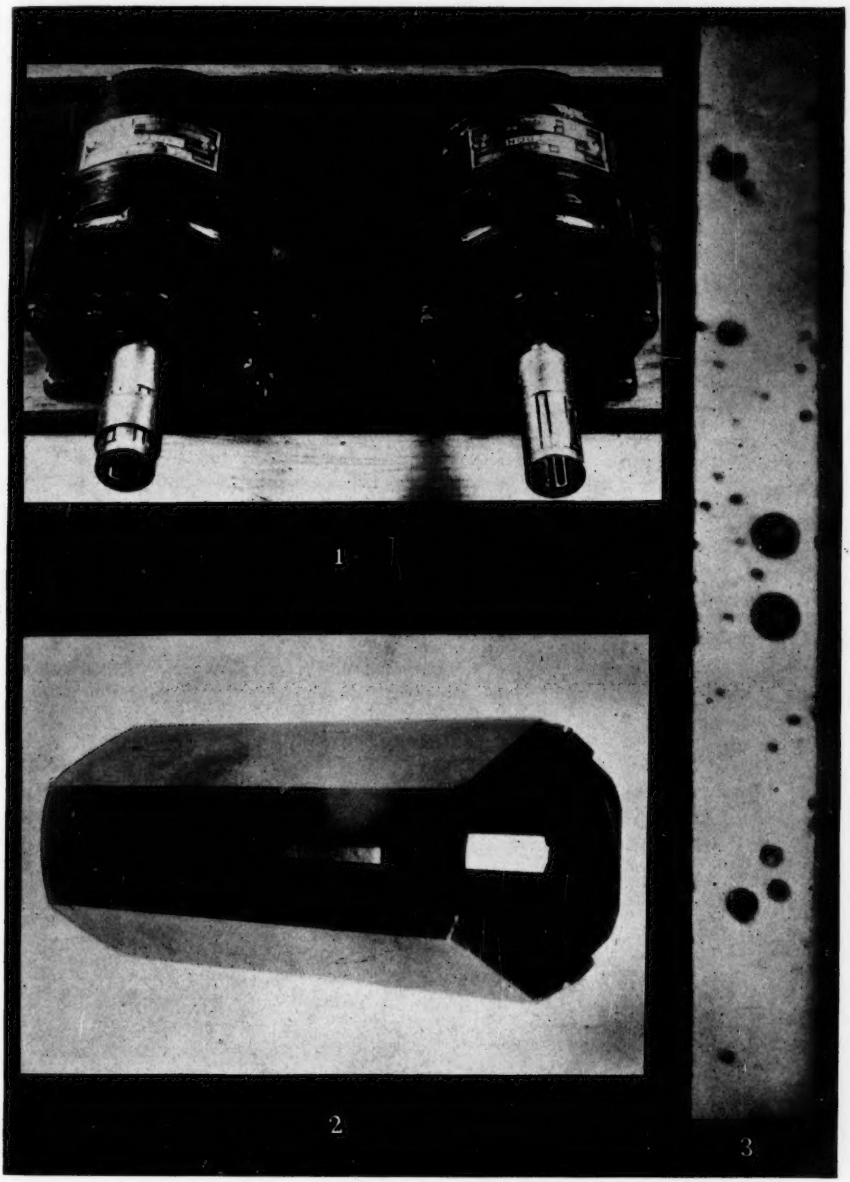

FIG. 1.—Device for spinning bottles, showing two kinds of chucks.
FIG. 2.—Brass cylinder with upper and lower slits.
FIG. 3.—Half-length of projected image of slit, showing surface and sub-surface colonies. Actual width of image. About 33 colonies per half-slit.

Tai and Van Heyningen.

REACTIONS OF THE RETICULAR TISSUES TO ANTIGENS.

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A NUMBER of studies have been made in recent years of the reactions of the lymphatic and other reticular tissues to foreign proteins and bacterial antigens. The results of such investigations have given rise to considerable controversy, partly over the cytological details of the reactions observed and partly over their interpretation and possible relation to the process of antibody formation. Ehrich and Harris (1942), Harris, Grimm, Mertens and Ehrich (1945) and Ehrich (1946) were principally responsible for the view that the tissue reaction to antigens was essentially a lymphocytic one, and Dougherty, Chase and White (1945) for evidence that the lymphocyte contained antibody, and that its release from the cell body was mediated by adrenal cortical hormone. An alternative view resulted from the work of Bing (1940), Bjørneboe and Gormsen (1943) and Fagraeus (1948), who described the formation of plasma cells in the lymphatic tissues both in animals following the injections of antigens and in man in conditions in which hyperglobulinaemia was present. Unfortunately, however, the dispute is not confined to these two sharply-conflicting views as to the nature and significance of the tissue reaction. The position is greatly complicated by the work of other authors who have published different descriptions and interpretations of the reaction, and also by the confusion of terminology and nomenclature which appears inevitable in any study of the lymphoid tissues. We have attempted, therefore, in this study to obtain the greatest possible precision in the description and definition of the cell types involved in these reactions in addition to any interpretation of their function.

MATERIALS AND METHODS.

Eighty adult male and female rabbits were employed for all the experimental investigations. The majority were about 2 kg. in weight, but smaller animals were sometimes used. The following antigens were employed :

Particulate Antigens.

Vaccines :

T.A.B., 2000 million organisms per ml. (1000 million *Salm. typhi*, 500 million *Salm. paratyphi A*, and 500 million *Salm. paratyphi B* per ml.) (Lister Institute heat-killed vaccine).

Mixed polyvalent pneumococcal, streptococcal and *H. influenzae*, 1600 million organisms per ml. (Parke, Davis).

* In receipt of a whole-time grant from the Medical Research Council.

Plague, 2000 million organisms per ml. (Parke, Davis).

Proteus vulgaris, an 18-hour broth culture of *Proteus vulgaris* killed by heating to 60° C. for 10 minutes.

Living organisms, an 18-hour broth culture of *Proteus vulgaris*.

Non-Particulate Antigens.

Normal horse serum (Burroughs Wellcome).

Hen's egg albumin, usually diluted with an equal volume of sterile, normal saline before injection.

Staphylococcus toxoid (Burroughs Wellcome Toxoid B).

Route of Injections and Dosage.

All antigen injections were given by the intravenous route into the marginal vein of the ear. The reasons for the selection of this route will be given later. Injections were either single, double or multiple at 3 to 4-day intervals, multiple injections being continued for a maximum period of 18 days. T.A.B. vaccine, plague vaccine and *Proteus vulgaris* vaccine were each given in doses of 1 ml., pneumococcal and streptococcal vaccine in doses of 2 ml.

Living cultures of *Proteus vulgaris* were injected at 3-day intervals, an initial dose of 0·1 ml. of an 18-hour broth culture being employed. In subsequent injections this dose was increased to 0·25 ml., 0·5 ml. and finally to 1 ml. This dosage produced no apparent deterioration in the general health of the animals.

Horse serum was given in a dose of 4 ml. and egg white (diluted as stated above) in a dose of 2 ml. Multiple injections of egg white were given to only a few animals owing to the development of severe anaphylactic symptoms. Staphylococcus Toxoid B was given intravenously, two injections of 1 ml. each

Estimations of antibody levels in the blood were performed on animals immunized with T.A.B. by the standard Widal technique, and on animals immunized with horse serum by the precipitin method of Dean and Webb (1926). A few estimations were made of the level of staphylococcus antihaemolysin by a technique recommended by Mr. H. Proom of the Burroughs Wellcome Research Laboratories.

Animals were killed at intervals after the first injection varying from 13 hours up to a maximum of 4 weeks.

Histological Methods.

All animals were killed with chloroform, and the spleen, lymph nodes, liver, lungs, kidneys and bone marrow fixed for 3 days or longer in the following mixture : absolute alcohol 60 ml., 40 per cent formaldehyde 12 ml., Aqua Dest. to 100 ml. With this mixture the fixation of rabbit tissues is superior to that given by formol saline, and permits the use of a greater variety of histological methods.

Paraffin sections of these organs were stained with Ehrlich's haematoxylin and eosin, with Pappenheim's stain and with a modification of this described later. Frozen sections were stained for reticulo-endothelial cells by a modification of the Weil-Davenport silver technique described by one of us (Marshall, 1946). In addition to wet-fixed material a number of air-dried imprint pre-

parations of spleen and lymph nodes were made immediately after the death of the animal and stained with Giemsa's stain.

We wish to emphasize that some method for the satisfactory demonstration of cytoplasmic basophilia is essential for the study of the cell reactions following administration of antigens. Pappenheim's stain or the modified method given later is the most suitable for this purpose, but good results may be obtained by Giemsa's stain, or by overstaining with haematoxylin followed by the minimum of eosin counterstaining. The ordinary haematoxylin and eosin preparation in which all haematoxylin staining is removed from the cytoplasm of the cell is of little value.

The following method has been found to give somewhat superior nuclear staining to Unna-Pappenheim's method alone.

Haematoxylin-Pyronin Method.

1. Place sections of formol-alcohol fixed tissues in Ehrlich's haematoxylin for 5 to 10 minutes.
2. Differentiate in acid alcohol until all cell cytoplasm is colourless.
3. After washing in tap water, rinse thoroughly in distilled water.
4. Place in Unna-Pappenheim's stain for 2 hours.
5. Dehydrate rapidly and mount in D.P.X. plastic mountant (B.D.H.).

CYTOLOGY OF NORMAL LYMPHOID TISSUES IN THE RABBIT.

It has been pointed out that the cytological changes following the administration of antigens involve both lymphocytes and plasma cells. Earlier work also dealt with the conception of the reticulo-endothelial system as a producer of antibodies. An account is therefore required of the normal cytology of these cell systems in the rabbit.

1. *Plasma cells* are sometimes completely absent from the spleen, particularly in very young animals, but scattered cells or small foci of mature cells are usually present. Occasionally, however, the rabbit shows a raised white cell count in the peripheral blood, and the spleen may then contain variable numbers of plasma cells of mature and immature type. In the popliteal and cervical lymph nodes of the normal rabbit, however, the medullary cords almost invariably contain numerous mature plasma cells. The mesenteric nodes, which comprise the largest single mass of lymphatic tissue, normally contain only scanty plasma cells in the medullary cords. It is clear, therefore, that in any observations of the reactions of plasma cells to the injection of antigens the main peripheral lymph glands of the rabbit will be of little value, and since injection into the area drained by the mesenteric nodes is technically extremely difficult, the spleen remains the most suitable lymphoid organ for study. For this reason all injections in this investigation were made by the intravenous route. In any case, it should be remembered that in an estimation of plasma cell increase the investigator is usually dealing with an increase in the number of cells normally present and not with complete new formation. The significance of a small increase in plasma cells should be regarded with caution.

2. *Lymphocytes.*—Little description is required of the lymphocytic elements of the spleen. These cells are normally present in the form of small and medium lymphocytes lying both diffusely in the red pulp and free in the sinuses. They

form large aggregations in the Malpighian bodies, and smaller collections surround the arterioles of the pulp. It is, however, important to point out that the Malpighian bodies are normally composed only of small and medium lymphocytes and of a few scattered cells with pale vesicular nuclei and scanty pale cytoplasm, the " primitive reticular cells " of Maximow. Mitoses are not present in these cells, and the so-called " germinal centres " or lymphocytopoietic centres are rarely present in the normal spleen.

3. *The " reticulo-endothelial system "* of the normal spleen of the rabbit requires considerably more description, since, as far as the authors are aware, no complete account of it exists. The usual methods of vital staining employed for the demonstration of this system are, in fact, incapable of revealing more than a small proportion of its normal extent, and, in addition, the morphology of the individual cells is not displayed at all except when they are lying free in the sinuses. For these reasons the silver impregnation methods used for the demonstration of the microglia which have been shown by Hortega (1921) and others (de Asúa, 1927 ; Dunning and Stevenson, 1934) to stain macrophages elsewhere in the body were employed solely in this study, and in this paper the term " R.E. system " will be confined to cells demonstrated by these methods.

EXPLANATION OF PLATES.

FIG. 1.—Normal reticulo-endothelial system of rabbit's spleen. Weil-Davenport method (modified). × 200.

FIG. 2.—Normal reticulo-endothelial system of rabbit's spleen. Weil-Davenport method (modified). × 600.

FIG. 3.—Development of mature plasma cells from primitive reticular cells in the rabbit's spleen, following intravenous antigen injection. Pyronin-methyl green.

- A. Primitive reticular cells.
- B. " Activated reticulum cell " showing early stage of cytoplasmic basophilia.
- C. Plasmablast.
- D. Immature plasma cells.
- E. Mature plasma cells.

FIG. 4.—Loss of lymphocytes from splenic pulp 13 hours after an intravenous injection of living *Proteus vulgaris*. Numerous polymorphonuclear leucocytes are present, some with pyknotic nuclei, but almost all lymphocytes have disappeared. H. & E. × 460.

FIG. 5.—Foci of developing plasma cells in splenic pulp after repeated injections of living *Proteus vulgaris*. Haematoxylin-Pyronin. × 185.

FIG. 6.—" Perifollicular envelope " (arrow) surrounding germinal follicle in rabbit's spleen after repeated antigen injections. H. & E. × 90.

FIG. 7.—Early stage of development of germinal follicle in rabbit's spleen 3 days after injection of *Proteus vulgaris* vaccine. Mitoses are present in primitive reticular cells. H. & E. × 500.

FIG. 8.—Germinal follicle in rabbit's spleen at height of development after repeated injections of living *Proteus vulgaris*. Numerous activated reticulum cells and large lymphocytes are present. H. & E. × 500.

FIG. 9.—Germinal follicle in rabbit's spleen at a late stage of development after repeated injections of a pneumococcal and streptococcal vaccine. Some mitoses are still present, but the majority of the cells are medium lymphocytes. H. & E. × 500.

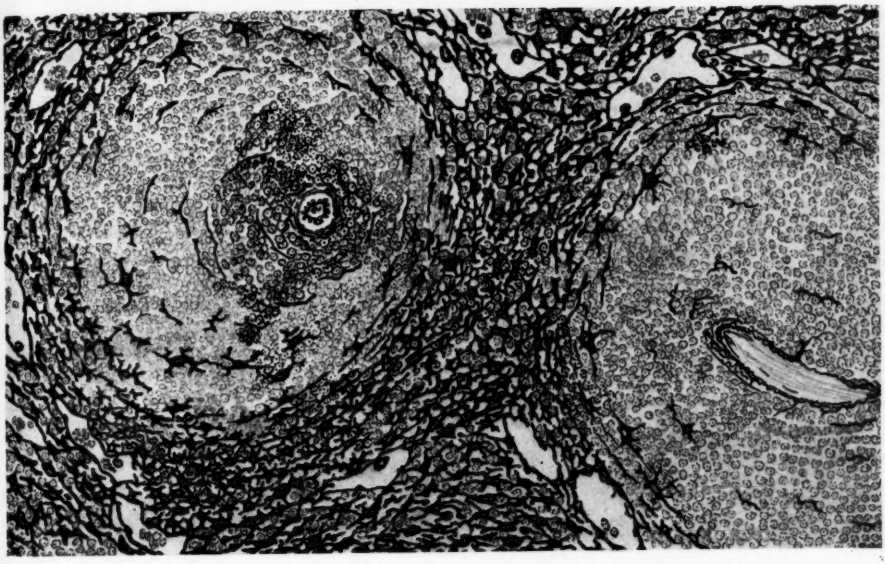
FIG. 10.—Lymphocyte, plasma cell and reticulo-endothelial cell granuloma in rabbit's lungs after repeated injections of living *Proteus vulgaris*. H. & E. × 185.

FIG. 11.—Granuloma in rabbit's liver composed of plasmablasts, immature plasma cells, and R.E. cells after repeated injections of living *Proteus vulgaris*. Pyronin-methyl green. × 590.

FIG. 13.—Popliteal lymph node of rabbit 7 days after injection of 2 mg. per Kg. of nitrogen mustard. The great majority of the lymphocytes of the node were destroyed. H. & E. × 90.

FIG. 14.—Same lymph node as Fig. 13. Undamaged plasma cells are seen in the medullary cords. H. & E. × 510.

FIG. 15.—Plasma cell proliferation in medullary cords of mesenteric lymph nodes of rabbit 9 days after 400 r of X-radiation. Pyronine-methyl green. × 410.



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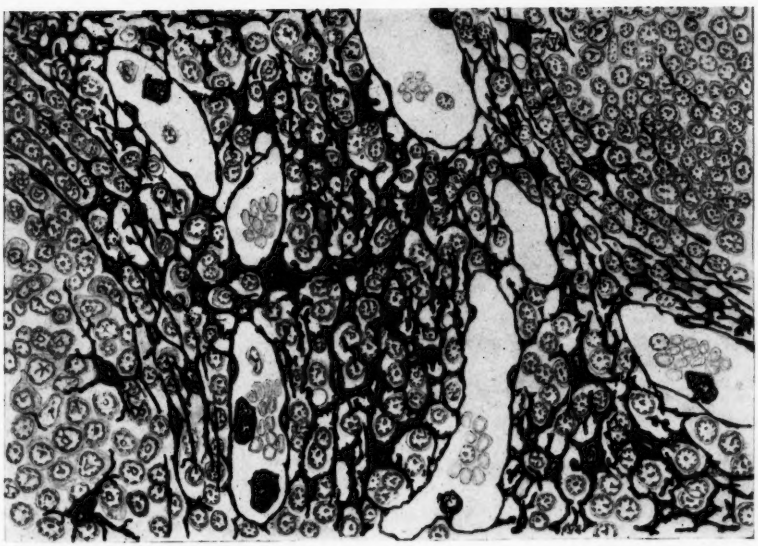

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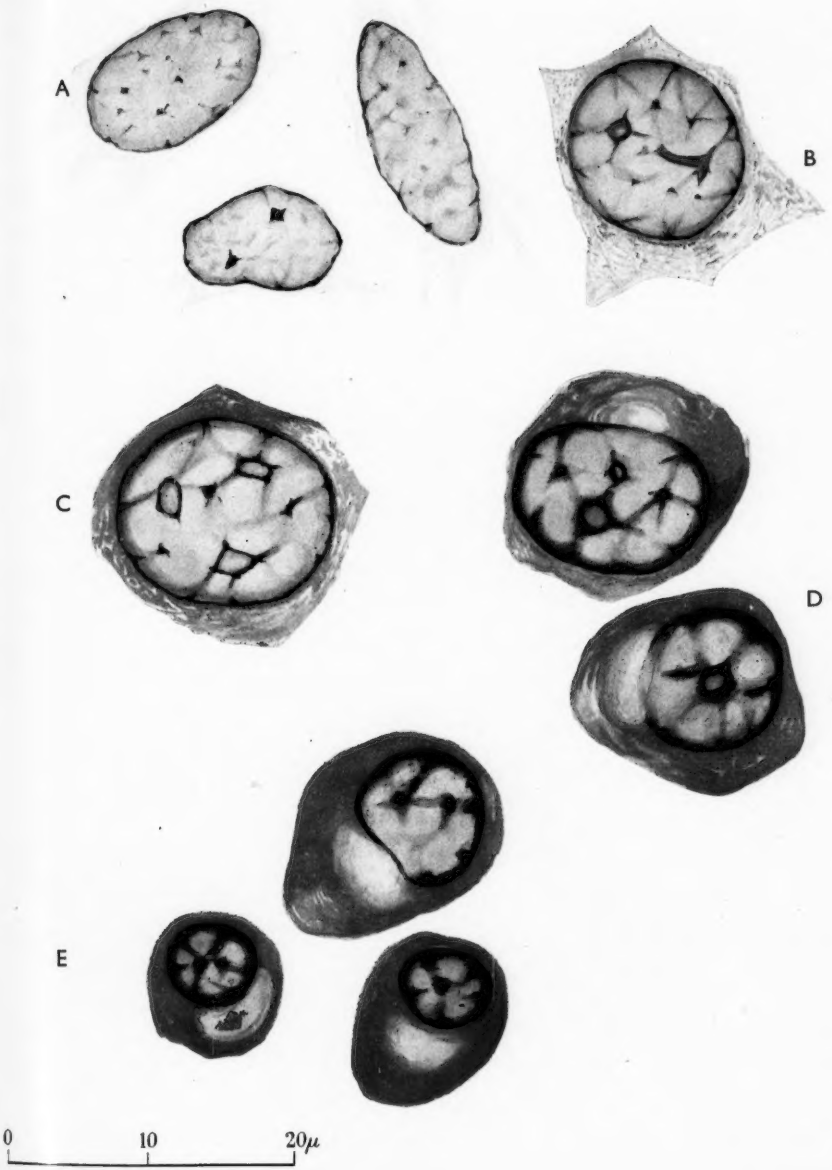

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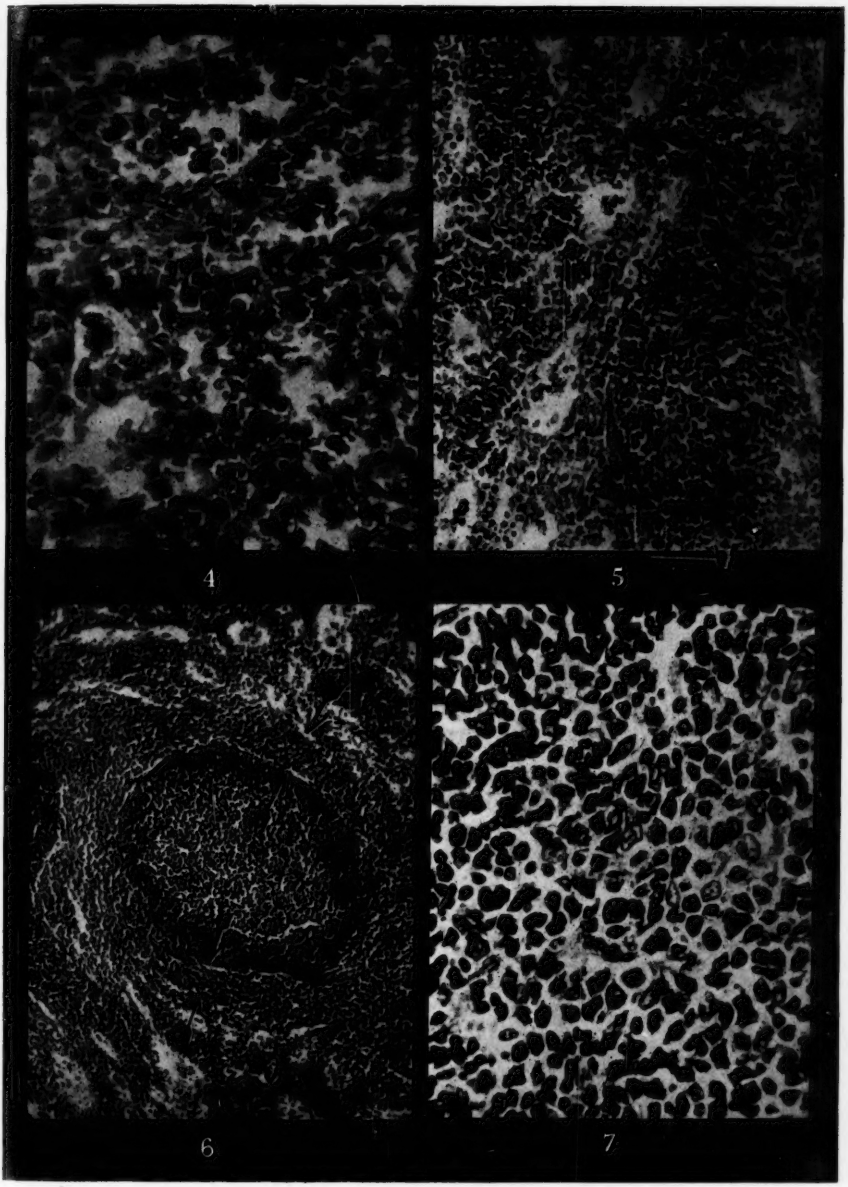

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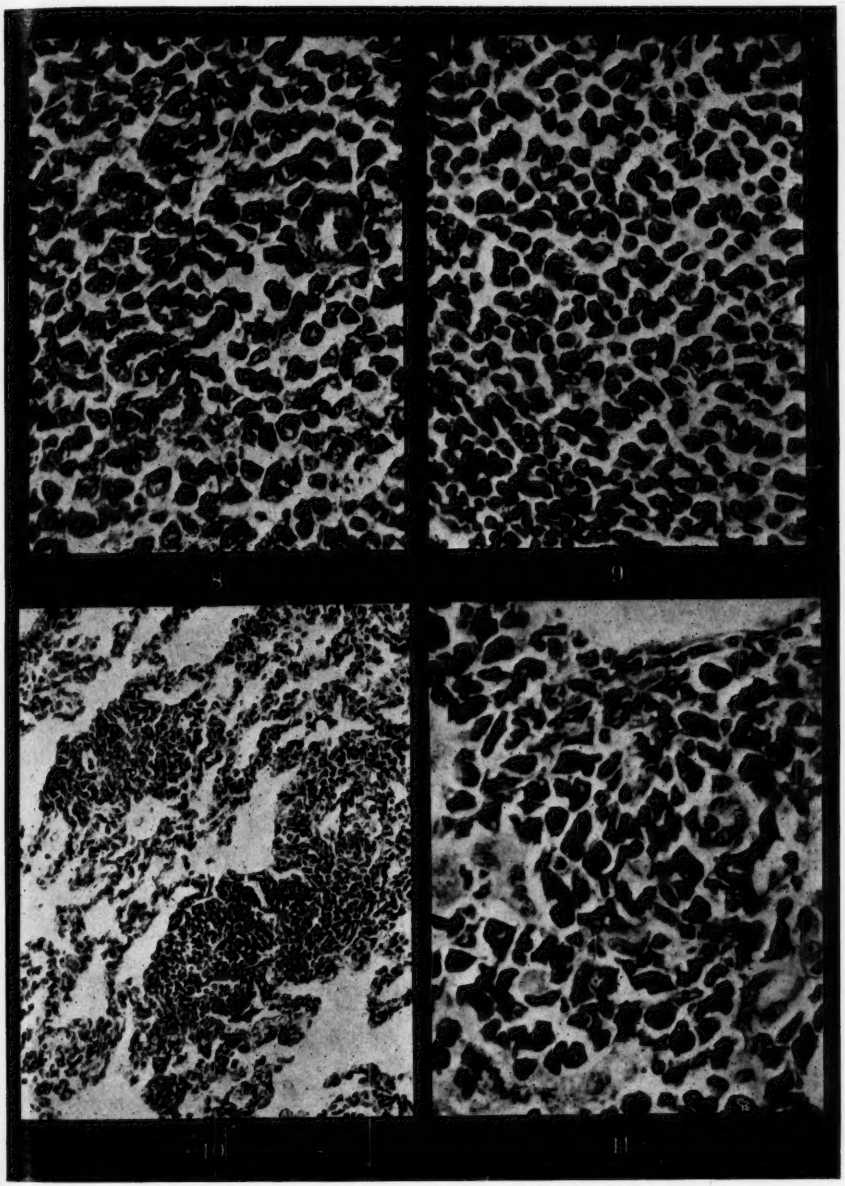

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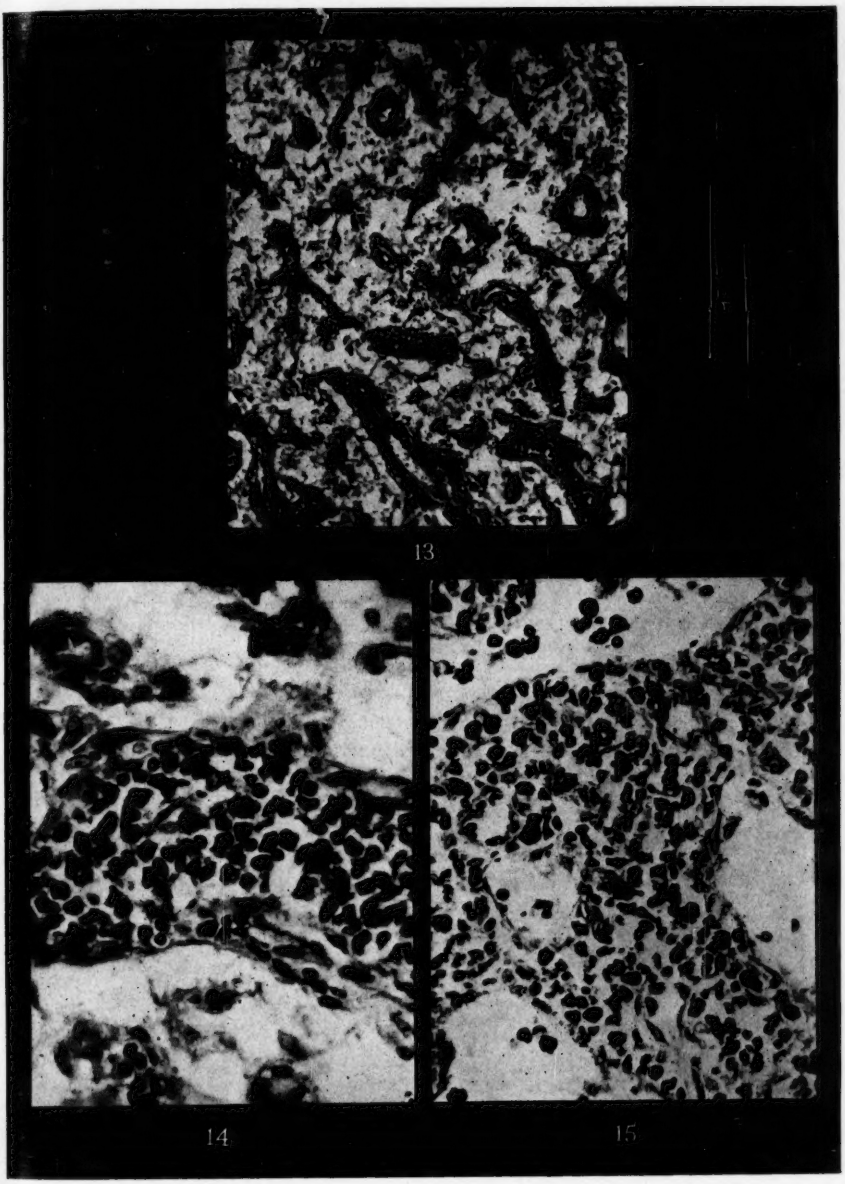

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Marshall and White.

One of us (Marshall, 1946) has previously described the appearances of some reticulo-endothelial cells demonstrated by these methods. While there is some doubt as to the position of the monocytes of the blood, it would appear that cells shown by the silver techniques correspond to the reticulo-endothelial system in the strict sense as defined by Aschoff (1924), that is, to the macrophage system of the tissues, which possesses both phagocytic ability and the property to store vital dyes. It may be pointed out, however, that while a cell which stores vital dyes is always demonstrated by the silver methods, the converse is not necessarily true in all organs. For this reason the use of silver staining methods on the spleen demonstrates a much larger number of reticulo-endothelial cells than may be seen after the most intense vital staining.

In recent years the term reticulo-endothelial system has come to be applied in a wider sense than that in which it was originally conceived, and by some American authors is used to describe almost any stem-cell of the haemopoietic or lymphoid tissues, as well as the macrophage system. It would seem from silver impregnation methods that there is no justification for this usage, and the reticulo-endothelial system appears sharply demarcated both by morphology and by staining reaction from the remaining cells of the lymphoid tissues.

In the splenic pulp the normal reticulo-endothelial system (Fig. 1 and 2) appears by the use of silver methods as a dense plexus of argyrophil cells which extend throughout the red pulp, scattered lymphocytes and haemic cells being present in the interstices between their processes. In the normal spleen these argyrophil cells are of four morphological types. The first type comprises the spheroidal macrophages lying free in the lumina of the sinuses, and requires no further description. In vitally-stained animals such cells are usually heavily stained.

The second type is composed of cells of an elongated spindle form, apparently with no lateral cytoplasmic branches which, lying end to end, constitute the lining layer of the sinuses, one surface of the cell thus being directly exposed to the blood stream. The impregnation of these cells is often difficult, and they are completely demonstrated only in the most successful preparations.

The third cell type comprises the bulk of the reticulo-endothelial tissue of the spleen, and is composed of cells lying in the stroma of the red pulp and possessing numerous irregular and coarse branching processes, which appear either to overlap one another, forming a tangled mass, or to join with the processes of neighbouring cells. In vitally-stained animals comparatively few cells of this type contain vital dye.

The fourth cell type is confined to the white pulp at the periphery of the Malpighian bodies, and is formed of cells with much finer and more extensive branching processes than the previous type. These cells closely resemble the microglia of the brain.

The normal Malpighian bodies are almost completely free of reticulo-endothelial cells and, even in those Malpighian bodies which possess active germinal centres, these cells are relatively scanty compared with those in the red pulp. Therefore little support is provided by the silver staining techniques for the "graveyard" theory of Ehrich (1946) that lymphocytes are destroyed in the reticulo-endothelial cells of the germinal centres.

In paraffin sections stained by ordinary histological methods the nuclei alone of resting reticulo-endothelial cells are shown. The nuclear characteristics

are variable, and the difficulty of identification on such characteristics alone is very great. In many instances the nuclei are similar to those of the microglia of the brain; that is, rod-shaped with a wrinkled nuclear margin and a moderately dense chromatin pattern. In other cells, however, the nuclei may be oval and larger than in the previous form and contain a fine vesicular chromatin pattern: this latter type appears to be common in the splenic pulp. A nucleolus is not prominent in these cells.

Nomenclature of Cell Types Present in Reactions to Antigens.

The descr ptions of cells in the following sections represent a selection of characteristics which are present in any group of cells together, but which cannot always be found fully developed in an individual cell.

Primitive reticular cell; "reticulum" cell.

In the resting state these cells (Fig. 3A) are present in the spleen in the normal Malpighian bodies, and apparently also in the immediate neighbourhood of the small arterioles of the pulp. They are prominent in active germinal centres.

The nuclei are oval or rod-shaped, the nucleoplasm colourless with very fine and scanty strands of chromatin, which at their intersections appear as small condensed dots. No definite nucleolus is visible in the majority of these cells. The outlines of the cell frequently appear ill-defined, and it sometimes appears to be connected with its fellows by syncytial processes. The cytoplasm shows no basophilia and, in contrast to the normal R.E. cells, is stained only a faint grey by microglial impregnation methods. Phagocytosis is not seen. Although the term reticulum cell is used by some authors as synonymous with a macrophage or histiocyte, it must be emphasized that these cells are different both in morphology, staining reactions, and behaviour from the reticulo-endothelial cells of the splenic pulp and elsewhere, when these are fully demonstrated by the silver impregnation techniques. The "undifferentiated mesenchymal cell" of Maximow (1924), however, which was described in many tissues, and particularly in the neighbourhood of small blood vessels, appears to be essentially similar, and no distinction will be made between it and the primitive reticular cells of the spleen.

"Activated Reticulum Cell."

This term has been given by us to cells which are not prominent in the normal spleen but which are numerous following antigen injection, both in the centres of active germinal follicles and in the pulp (Fig. 3B). They are similar to cells commonly composing certain of the reticulum cell sarcomas in man. Their morphology is, in general, similar to the primitive cell, though somewhat larger in size and showing a varying degree of cytoplasmic basophilia; this is often present in the early stages only in the form of fine scattered basophilic particles, which later become confluent and fill the cytoplasm, defining its outline. The nucleus is oval or round, and made up of fine strands of chromatin with one or two prominent basophilic nucleoli, sometimes round, sometimes of irregular elongated form. The cells show no evidence of phagocytic activity, and the cytoplasm is impregnated only a faint grey by microglial methods.

Plasmablast.

An oval or occasionally round cell 15 to 20 microns in diameter in fixed tissues (Fig. 3C). The nucleus is situated in the centre of the cell, and closely resembles that of the haemocytoblast of the blood-forming organs; that is, round, leptochromatic, and possessing multiple basophilic and usually round nucleoli.

An intense degree of cytoplasmic basophi ia is present, which, however, is not completely uniform throughout the cell. Apparently exactly similar cells may be seen in the marrow in some cases of plasma-cell myeloma, and in imprint-preparations stained by Giemsa's method they closely resemble the cells described by Moeschlin (1940) in the bone marrow and lymph nodes of cases of german measles. Karyokinetic division is common in these cells.

Immature plasma cell.

In these cells further maturation has occurred (Fig. 3D). The nucleus has become eccentrically placed in the cell body, and shows a condensation and thickening of the chromatin strands and a slight reduction in diameter. A clear oval vacuole 3 to 5 microns in the longer diameter is present in the cytoplasm adjacent to the nucleus. The cytoplasmic basophilia is as intense as that of the plasmablast, and is uniformly distributed. Mitotic division is also common in these cells.

Plasma cell.

The mature plasma cell is too well known to need detailed description. We have confined the term to cells of typical morphology with a "clock face" arrangement of the nuclear chromatin, and to the more numerous cells in which there is a less regular arrangement of the chromatin (Fig. 3E).

REACTIONS OF THE SPLENIC TISSUES TO ANTIGENS.

General Features of the Reactions.

The reactions are of a uniform type for all antigens tested, and the same cell elements are involved in all cases. Differences in degree, however, exist in the reactions produced to different antigens both in the intensity of the whole reaction and in the prominence of different cellular elements in it. Allowance having been made, however, for these differences, the response to antigens may be divided into reactions of the primitive reticular cells of the pulp, reactions of the same cells in the Malpighian bodies, and the reaction of the argyrophilic cells of the reticulo-endothelial system. The changes taking place in certain of these cells represent the proliferative part of the whole reaction; in addition, certain changes of an apparently purely destructive and inflammatory nature take place in the period immediately following the injection of the antigen.

I. Early Reactions to Antigen Injection.

Following intravenous injection of an antigen two rapidly-occurring changes may be seen in the splenic tissue. The first, which is particularly marked with bacterial antigens, consists of a diffuse infiltration of the red pulp with polymorphonuclear leucocytes. The majority of the cells lie free in the sinuses, but

many may be present in the red pulp and sometimes in small clumps among the lymphocytes of the Malpighian bodies. This change occurs within 8 hours of the injection of the antigen and has usually disappeared within 2 to 3 days. Many of the polymorphonuclear cells appear to degenerate rapidly and to be phagocytosed by the splenic macrophages. The remnants of the nuclei of these phagocytosed cells may be seen within macrophages for a period of several days. Many authors assume that such intracellular fragments in the splenic tissues usually represent the remains of lymphocytes; this does not appear by any means to be always true.

The second change immediately following antigen injection occurs within 12 hours, and appears to persist for 36 hours or slightly longer. This change consists of a disappearance of small lymphocytes from the sinuses and red pulp of the spleen (Fig. 4). After the injection of living *Proteus* this change was particularly well marked, and in such animals an almost complete disappearance of the small lymphocytes normally diffusely scattered in the pulp of the spleen had occurred. Yet, apart from the occasional polymorphonuclear infiltrations previously described, little change appeared to have taken place in the lymphocytes of the Malpighian bodies.

It is uncertain whether this loss of lymphocytes is produced by their destruction or mass emigration from the splenic tissues. Evidence of destruction in the form of pyknotic or phagocytosed cells is not very conspicuous, and it is probable that the latter process accounts for the bulk of the change seen.

II. Later Reactions in the Splenic Pulp.

These reactions may be divided into two parts, a proliferation of activated reticulum cells in the pulp which differentiate into a number of cell types with the eventual formation of plasma cells, and a reaction involving the reticulo-endothelial cells.

(a) *Reaction of activated reticulum cells.*—This may be seen in its earliest stage about 3 to 4 days following a single injection of an antigen (primary response). At this time a number of large basophilic cells, resembling the activated reticulum cells previously described, appear in small foci diffusely throughout the pulp of the spleen, to some extent in the sinus walls, but more particularly in the immediate region of the arterioles of the pulp. Mitoses may be present in such foci, and at a slightly later stage the majority of the cells in them consist of basophilic cells resembling those which we have classed as plasmablasts. It would seem that these correspond closely to those cells described by Rich, Lewis and Wintrobe (1939) as " The acute splenic tumour cell," by Hewer (1930) as myeloblasts, by Goldzieher (1927) as histiocytes, and by Fagraeus (1948) as " transitional cells." It must be emphasized that no certain opinion can be given as to the nature of these cells at this stage, and the term " plasmablasts " is only used in view of the direction of their later differentiation.

If repeated injections of an antigen are given at 3 to 4 day intervals, the foci of plasmablasts increase rapidly, mitoses being frequent, and may in extreme cases become confluent to occupy the greater part of the pulp of the spleen (Fig. 5).

Under such conditions rapid differentiation of the cells takes place, and by the 12th or 13th day from the first injection the proliferating foci are composed of a mixture of cell types. In this proliferation (secondary response) multipli-

cation appears to be mainly by mitotic division at the plasmablast level and there seems to be little further production of activated reticulum cells. The plasmablasts may still be in the majority, but considerable maturation has occurred in many of these cells and forms with eccentrically-placed nuclei, immature plasma cells are present in numbers, and in some cases maturation has proceeded to the formation of mature plasma cells. Many of the cells present are not classifiable precisely as plasmablasts, immature plasma cells or mature plasma cells, but are intermediate forms. At a still later stage, after 3 to 4 weeks of repeated injections, very few immature cells are present, the reaction being composed of mature plasma cells only.

At the margins of the Malpighian bodies a broad zone of activated reticulum cells containing many mitoses is frequently seen in immunized animals (Fig. 6); this zone corresponds to the " perifollicular envelope " described by Krumbhaar (1948) in the spleen of rats.

In animals killed after prolonged immunization many mature and immature plasma cells may be seen in this zone. As will be described later, this area also contains many true reticulo-endothelial elements.

Effect of anti-histamine drugs on plasma cell formation.

Dougherty (1948) has stated that the formation of mature plasma cells is probably an allergic phenomenon due to an antigen-antibody reaction occurring in the cytoplasm of a lymphocyte. This effect could conceivably result from the liberation of histamine consequent upon such an antigen-antibody reaction, and it was, therefore, decided to study the effect of anti-histamine drugs on the formation of plasma cells to an antigen.

Method.—Eleven rabbits were used. All animals received a total of 16 ml. of a mixed pneumococcal and streptococcal vaccine (Parke, Davis), given in 6 divided doses at 3-day intervals by intravenous injection. Four animals were reserved as controls and received no further treatment. Of the remaining 7 animals, 4 received an intravenous injection of " Benadryl " hydrochloride (Parke, Davis) at a dosage of 5 mg. per kg. body weight 5 minutes before the injection of each dose of vaccine. The remaining 3 animals, in addition to injections of " Benadryl," each received a total of 1·8 ml. of histamine azo-protein (" Lertigon " Parke, Davis) in 3 divided doses by subcutaneous injection before the commencement of vaccine treatment. A further injection of 1 ml. of histamine azo-protein was given 8 days after the start of immunization. All animals were killed 5 days after the last injection of vaccine, and the spleen, liver and lymph nodes excised for histological examination. The dosage of " Benadryl " employed appeared to be near the maximum tolerated dose, and slight convulsions occurred in some of the treated animals.

Result.—The spleens of both control animals and those treated with anti-histamine substances contained numerous mature plasma cells, and no difference could be detected between the control and treated groups. Germinal follicles were also present in the spleens of both groups.

III. Reactions of the Malpighian Bodies to Antigens.

The proliferative changes involving the Malpighian bodies began in a period of three to four days following the intravenous injection of an antigen. As with the plasma cell reaction, they were produced by all antigens employed.

The earliest change is the appearance of mitotic figures in the primitive reticular cells of the Malpighian body (Fig. 7), and in the course of the next three to four days numerous large stellate cells with basophil cytoplasm, which resemble the cells described as " activated reticulum cells," appear in the area. At this stage these cells appear identical with those previously described as occurring in the splenic pulp. Many mitoses are present in the activated reticulum cells, and smaller cells resembling large lymphocytes appear intermingled with them (Fig. 8). At this stage the proliferating tissue forms a distinct pale area, the so-called " germinal centre," often eccentrically placed in the Malpighian body and surrounded by a rim of darkly-staining small lymphocytes. After a single injection of an antigen only a proportion of the Malpighian bodies are involved in this reaction, and even after numerous injections a few remain unaffected.

With a number of injections of antigens the subsequent development of the cells in the germinal centres appears to be as follows: The mitoses decrease in number, the " activated reticulum " cells disappear from the centres; in a period of three to four weeks the centres consist of medium lymphocytes (Fig. 9), and it becomes increasingly difficult to discern the margin between the germinal centre and the small lymphocytes comprising the remnants of the original Malpighian body. It has been stated by Rich *et al.* (1939) that the origin of the large basophilic cells seen in the pulp of the spleen after antigen injection was by migration of reticulum cells formed in the germinal centres. As stated in the previous section, however, such cells appear diffusely in the splenic pulp, and further, at a time when the germinal centres show only commencing activity. We have observed no evidence in our material of a migration which would provide a source for the basophilic cells (" activated reticulum " cells) involved in the early stages of plasma cell formation. At the height of the development of the germinal centre, however, such cells may be seen outside the centre, and intermingled with the small lymphocyte zone surrounding it. It is possible that these cells may have migrated from the follicle.

After the intravenous injection of antigens, the formation of germinal centres is confined to the spleen, but their formation in lymph nodes after subcutaneous or intramuscular injection has been described by a number of authors. It has been stated that all antigens produced the formation of germinal centres; it is our impression, however, that they reach their greatest development when bacterial antigens are employed: both egg-white and horse serum seem to produce a considerably less intense reaction.

While the germinal centres which were produced closely resembled the Flemming's centres seen in human lymph nodes (reactive follicular hyperplasia of Robb-Smith, 1938), the cytology was not identical, as macrophages were present in smaller numbers and the characteristic " starry sky " appearance seen in human material was not well marked.

IV. Reaction of the Reticulo-endothelial System of the Spleen to Antigens.

The importance of the reticulo-endothelial system in antibody formation has been stressed by many authors. The changes observed by us in this system in the spleen after antigen injection, however, do not appear to be of a striking nature. In contrast to the plasma cell reaction in the red pulp, and the formation of germinal follicles, it is doubtful whether any proliferation of the reticulo-endothelial system takes place, the major part of the enlargement of the spleen

that occurs being provided by the former two reactions. While, however, no demonstrable proliferative changes take place, alterations may occur in the morphology of the reticulo-endothelial cells of the splenic pulp. The number of finely branched argyrophil cells seen in the normal spleen becomes lessened, and more coarsely branching cells of an amoeboid type appear; associated with these an increase in the number of free rounded argyrophilic cells in the lumen of the sinuses may occur.

In some cases amoeboid cells of this type come to constitute the great majority of the reticulo-endothelial cells of the pulp.

Such changes resemble in morphology the transitions occurring in the brain following injury, between the resting microglia and the compound granular corpuscles.

A further change was seen in the "perifollicular envelopes" previously described: while the majority of the cells in these structures appeared to be activated reticulum cells, a considerable proportion were true reticulo-endothelial cells of the resting type with fine branching processes. Such cells, however, did not extend into the germinal follicle proper, which contained only scanty reticulo-endothelial elements. As the "perifollicular envelope" proliferates following the administration of antigens, it would seem that some true multiplication of reticulo-endothelial cells does occur in this structure.

Fagraeus (1948) in her work on the plasma cell response to antigens has described the origin of plasma cells from the reticulo-endothelial system. If this process occurs, it should be demonstrated in splenic tissues by the use of microglial impregnation methods. In our animals, however, in spite of the fact that great differences in morphology and staining reaction exist between the reticulo-endothelial cell and the plasmablast when silver staining methods are employed, no evidence of transitional forms could be observed following the injection of an antigen.

Reactions to Intravenous Antigen Injection in Other Organs.

Apart from the cytological changes described in the spleen, cellular reactions of a somewhat similar type may occur in other organs following intravenous injection of antigens. These changes were observed only after multiple injections, and were produced only by horse serum, polyvalent pneumococcal vaccine, and living *Proteus vulgaris*, out of the total number of antigens employed.

In order of frequency and of intensity of the reaction, the lungs, liver, bone-marrow and kidneys were involved.

In the lungs (Fig. 10) foci of plasmablasts, plasma cells, small lymphocytes and reticulo-endothelial cells were observed, both surrounding the pulmonary vessels and also as scattered foci in the alveolar walls, sometimes leading to diffuse thickening of the alveolar septa.

In some animals numerous plasma cells were seen in the lumina of the alveolar capillaries. The lymphocytes were scattered diffusely among the other cell elements, and no structures resembling germinal centres were present. The reticulo-endothelial cells were mostly of the branching "microglial" type, and resembled those normally found in the lung. In a few cases the cell foci present attained a large size, and were visible to the naked eye as minute grey flecks scattered over the cut surface of the lung.

In the liver similar lesions (Fig. 11), consisting of foci of small lymphocytes

mixed with plasma cells and Kupffer cells, were seen in the lumina of the sinusoids and adjacent to the wall of the portal veins. In addition to focal accumulations of this type, scattered plasma cells could be seen lying separately in the sinusoids.

The lesions in the liver did not in any instance attain the size and frequency of those in the lung.

In the bone-marrow only a few focal accumulations of plasma cells were seen in heavily-immunized animals, and similar small plasma cell foci were present in the connective tissue of the renal pelvis in a few animals.

No other definite lesions were observed in any other tissue examined, neither the lymph nodes nor the thymus showing any well-marked alteration.

The acute vascular lesions described by a number of authors (Rich and Gregory, 1943; Ehrich, Seifter and Forman, 1949) following large doses of horse serum were not observed in any of our animals, probably because the dosage employed was insufficient.

Effect of Lymphocyte Destruction Upon Resting Antibody Levels.

It has been claimed by some authors (Dougherty *et al.*, 1945) that injection of adrenal cortical hormone in previously-immunized animals causes a rise in the resting antibody level in the course of a few hours. The rise in antibody is accompanied by destruction of lymphocytes in the spleen and lymph nodes of the injected animal. It was considered by these authors that such observations provided evidence for the formation and storage of antibody by the lymphocyte.

It has been shown (Kindred, 1947) that nitrogen mustard is a potent lymphocyte-destroying agent, and we have employed this substance in an attempt to confirm the observations of Dougherty *et al.* (1945).

Methods.

Twelve adult rabbits were used for this experiment. All animals were immunized with either one or two intravenous injections of 1 ml. of T.A.B. vaccine, and were then left for a period of three to four weeks after the last

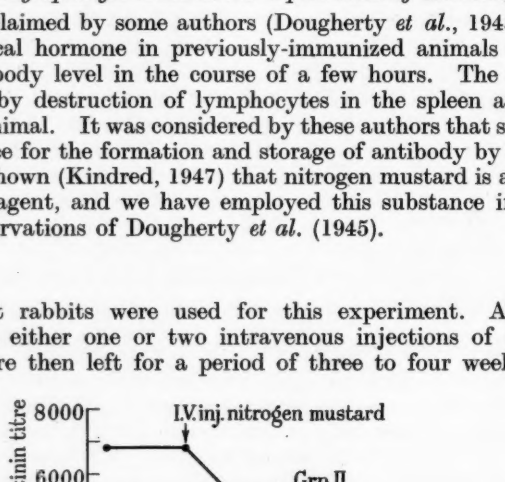

FIG. 12.—Effect of intravenous injection of a varying dosage of nitrogen mustard upon the agglutinin titres to *Salm. typhi* H. Each curve represents the mean titre of a group of 3 animals.

Group I. 0·4 mg. per kg. body weight.
" II. 0·6 " "
" III. 0·8 " "
" IV. 1·6 " "

injection. Samples of blood were then taken from all animals for estimation of the agglutinin level immediately before an injection of nitrogen mustard hydrochloride (Boots) in saline was given intravenously. A variable level of dosage of this substance was employed, the animals being divided into four groups each of three rabbits, the groups receiving a dosage of 0·4, 0·6, 0·8 and 1·6 mg. per kg. body weight respectively.

Samples of serum were withdrawn from the animals at periods of from 4 to 90 hours following the injection, the times of withdrawal being varied in each group in order to detect, if possible, rises in agglutinin titre of short duration.

The animals were killed at the termination of the experiment, and their lymph nodes, spleens and bone-marrow examined histologically.

Estimations of the level of agglutinins to *Salm. typhi* "O" and "H" were performed by the standard Widal technique.

Results.

As can be seen from the chart, there was no evidence of any rise in antibody titre in any of the 4 groups of animals following the injection of nitrogen mustard. "H" agglutinins only are shown, but similar results were obtained with the "O" curves. The falls in titre that occurred in two groups can probably be disregarded as being within normal experimental error. White cell counts of the peripheral blood, including absolute lymphocyte values, were done on all animals at the time the sera were taken for estimation of agglutinins. It was found that the lymphocyte level fell to between 30 and 50 per cent of its former value within 5 to 24 hours after injection. The histological changes in the lymphatic and myeloid tissues examined showed an increasing degree of destruction of small lymphocytes and myeloid tissue as the dosage was increased. The most susceptible cells were the small and medium lymphocytes and the immature cells of the leucocytic and erythroblastic series. Large lymphocytes appeared considerably more resistant. There was little evidence of damage to the reticulo-endothelial system. When active germinal follicles in the spleen were present, they appeared to be somewhat reduced in size from those in untreated animals, but were not completely destroyed. Both plasma cells and plasmablasts appeared to be completely resistant to the dosage employed, a feature that could be seen in the spleens of treated animals, but most strikingly in the popliteal lymph nodes, where the mature plasma cells normally present in the medullary cords of the node remained apparently untouched in the presence of almost complete destruction of the small lymphocytes of the remainder of the gland (Fig. 13 and 14).

Influence of X-radiation on the Formation of Plasma Cells.

It has been stated by Parsons (1943) that X-radiation of the lymph nodes of mice gives rise in a few days to a massive plasma cell proliferation. Similar plasma cell formation was described by Ross (1932) in man following exposure to radium, by Liebow, Warren and Delauney (1949) in man after exposure to radiation in atomic explosions, and by Thomas and Bruner (1933) in rats after chronic radium poisoning. It was decided to attempt a similar experiment in rabbits to see whether the reaction produced resembled that caused by antigens. A further object of the experiment was to test the resistance of the plasma cells normally found in the popliteal lymph nodes to X-radiation.

Methods.

Eight adult rabbits were employed. Each animal was given a single tissue dose of 400 r to the lower chest, abdomen and hindquarters. The animals were subsequently killed at intervals of from three to fifteen days following radiation.

Results.

The spleen and lymph nodes in the radiated animals showed a marked degree of destruction of small and medium lymphocytes. In the spleen, apart from the destruction of the Malpighian bodies and white pulp, there was little other change seen ; a few plasma cells, however, were present in some animals. No change was seen in the liver. On the other hand, the mesenteric nodes of all animals examined showed not only great destruction of lymphocytes, but the presence also of numerous plasma and immature plasma cells in the medullary cords, many of the cells being in mitosis (Fig. 15). These were in excess of the numbers normally observed in the mesenteric nodes. In the popliteal glands, while there was little evidence of plasma cell proliferation, the plasma cells normally seen in the medullary cords showed no evidence of damage from radiation, in contrast to the lymphocytes in the cortex of the nodes. This resistance of the plasma cells to radiation appeared to be similar, therefore, to their resistance to the effects of nitrogen mustard.

DISCUSSION.

It has been shown that, following the intravenous injection of an antigen, two types of cellular proliferation take place in the spleen, and that these changes may be associated with cellular proliferations in other organs. In both the splenic reactions the initial change is a multiplication of reticulum cells leading, in one case, to the formation of plasma cells in the red pulp, and, in the second, to the formation of lymphocytes in the germinal centres. The changes in other organs (liver, lungs and kidney) are of a similar nature in that a mixture of plasma cells and lymphocytes is formed, the latter being produced, however, without the formation of actual germinal follicles. In addition to these reactions, certain changes in the reticulo-endothelial system have been described which do not, however, appear to be of a proliferative nature.

If, therefore, as suggested by Burnet (1941), the production of immune bodies is accompanied by the multiplication of a cell system, it is probable that the formation of antibodies is accomplished by either the plasma cell portion of the reaction or by the proliferation in the germinal follicles which appears to lead to the formation of lymphocytes.

It may be pointed out that, although in the two reactions the end product is the formation of plasma cells and lymphocytes respectively, the formation of antibody cannot be the sole prerogative of either of these two cells in the mature state. As immune bodies may be detected in the blood of a rabbit three to four days after intravenous injection, the formation of such antibody, cannot be due to the finally differentiated forms of the cellular reaction. At the time of the first formation of antibody the cell proliferation has not progressed far beyond the stage of the " activated reticulum " cell or plasmablast. One may not, therefore, speak of plasma cells or lymphocytes producing antibody, but only of the whole plasma cell or lymphocyte reaction resulting in its formation. Even

at the height of the antibody response the reactions are still composed of mixed cell types, of plasmablasts, immature plasma cells, and plasma cells in one case, and reticulum cells, large and medium lymphocytes in the other.

Further, in the primary response to an antigen relatively few mature plasma cells are formed, differentiation to mature forms only proceeding rapidly with subsequent injections. It has been suggested in view of this (Dougherty, 1948) that the formation of mature plasma cells is in fact an allergic phenomenon due to an antigen-antibody reaction taking place in the cytoplasm of a lymphocyte. Apart from the failure of anti-histamine drugs to influence plasma cell production which we have described, this view is rendered improbable, both on account of the presence of a sequence of primitive plasma cell types which do not resemble lymphocytes, and also in view of the active plasma cell formation which occurs in multiple myelomatosis in the absence of any known antigen or antibody.

We have already stated that the function of the germinal centres in the spleen appears to be the formation of small lymphocytes, and they are in fact lymphocytopoietic centres. In recent years, following the work of Hellmann and White (1930), Heiberg (1926) and of Österlind (1938), it has become customary to term these structures " reaction centres " and to consider them to be unassociated with increased lymphocyte production. This term adds little to knowledge, as both the nature and function of the reaction and the causes exciting it appear equally obscure. It also appears doubtful whether the terms " reaction centre " and " germinal centre " are completely synonymous. Maximow and Bloom, (1942) distinguished between centres actively forming lymphocytes and those composed mainly of reticular cells and macrophages. We have seen that, in accordance with the work of other observers, germinal centres are regularly produced by antigen injection ; they may, however, also be produced by chemical agents (Ehrich, 1931), and may further occur in a confusing variety of conditions in man. Thus they may be present in the thymus in some cases of myasthenia gravis (Bratton, 1948), in the thyroid gland in Hashimoto's and Graves' disease, and apparently in the skin in the condition known as lymphocytoma cutis (Epstein, 1935). It is accordingly difficult to accept the view that the germinal centre represents solely an antibody forming structure. While the cytology of the germinal centre indicates that the function of the structure is to produce lymphocytes, this does not require acceptance of the old view that it is a normal source of the lymphocytes of the blood and lymph nodes. Germinal centres are rare in normal animals, and are stated not to occur in the embryo or in animals kept in a sterile environment (Glimstedt, 1936). However, this does not prove that local production of lymphocytes may not occur through the action of germinal centres where there is a special need, and it has been shown, both in our material and by other authors, that, following antigen injection, the germinal centre reaction is an essentially local one confined to the site of lodgement of the antigen, either in the regional lymph nodes following subcutaneous injection, or in the spleen following intravenous administration. It has further been observed in our material that a loss of small lymphocytes occurs in the spleen shortly after the intravenous injection of an antigen ; a similar loss of lymphocytes in a lymph node following injection of an antigen has been observed by Habel, Endicott, Bell and Spear (1949), and in the spleen by Delaunay, Lebrun and Delaunay (1949).

We consider, therefore, that the formation of germinal centres following antigen injection represents an essentially reparative process directed towards the re-stocking of the tissue with lymphocytes. The view that the small lymphocytes produced by the germinal follicles could be the source of antibody is not possible, as mature cells are produced only after the formation of antibody has reached its height.

This does not, however, eliminate the possibility that the germinal follicles formed after antigen injection might also be responsible for the formation of antibody during their active stage of development, and it is hoped to provide further evidence on this point in a later publication. The direct formation of antibody by small lymphocytes, however, is rendered additionally improbable by the failure to obtain evidence of antibody release after destruction of these cells by nitrogen mustard. This result is in agreement with the findings of most workers who have attempted to confirm the original report of Dougherty and White. Amongst others, failure to obtain release of antibody after both heavy X-radiation and injection of adrenal hormone was observed in cats by Craddock, Valentine and Lawrence (1949), in rabbits following X-radiation by Craddock and Lawrence (1948), and in man following A.C.T.H. by Herbert and De Vries (1949). We have shown, however, that plasma cells are highly resistant to both X-radiation and nitrogen mustard and, therefore, if these cells contain antibody, failure to obtain its release after the use of such agents is to be expected.

The significance of the apparent stimulation of plasma cells in the mesenteric nodes by X-radiation is more difficult to assess. It is of interest in this connection that Dougherty and White (1945) claimed that plasma cell production occurred in the rabbit's spleen following the injection of adrenal cortical hormone. The action of both X-rays and adrena cortical hormone is to cause lymphocyte destruction ; antigens also appear to have the property of causing loss of lymphocytes from the spleen or lymph nodes ; whether there is any causal connection between the lympholytic action of such agents and their powers of stimulating plasma cell production requires further investigation.

Fagraeus (1948) described the transformation of the macrophage which ingested the antigen into the developing plasma cell ; we have been unable, however, to obtain histological evidence for this process. The final solution of this problem would appear to require the use of a marked antigen which could be traced in the reticular tissues for a period of some days or weeks after its ingestion.

SUMMARY.

The normal cytology of the reticular tissue of the rabbit spleen has been described.

An investigation has been made into the cell changes produced in the spleen and other tissues of the rabbit by the intravenous injection of antigenic substances, the effect of nitrogen mustard upon antibody levels, and the effect of X-radiation upon plasma cells in the lymphatic tissue. It has been shown that :

(1) The intravenous injection of various antigens in the rabbit produces a stimulation of primitive reticular cells which leads to the formation of plasma cells in the spleen, lungs, liver and bone marrow and to the formation of germinal follicles in the spleen only.

(2) The function of the germinal follicles is considered to be the formation of lymphocytes.

(3) Destruction of lymphocytes by nitrogen mustard does not produce a release of antibody in immunized animals.

(4) Plasma cells are highly resistant to both nitrogen mustard and to X-radiation and appear to be stimulated by the latter agent.

The significance of these findings is discussed.

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MITOTIC ACTIVITY IN THE SHOCK STATE.

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RECENT work has established the close dependence of mitotic activity on an adequate supply of glucose (Bullough, 1949*b*). In conditions of experimentally-induced hyperglycaemia the mitosis rate is high, while in conditions of experimentally-induced hypoglycaemia it is low. In the course of our work on nucleotide shock the fact that sugar metabolism was profoundly affected in the general reaction to tissue injury was confirmed and amplified (Green and Stoner, 1950; Green, Stoner, Whiteley and Eglin, 1949). It was of interest therefore to see whether the hyperglycaemia of the shock state was reflected in a raised mitosis rate, particularly as the general depression of metabolism, one expression of which is the fall in tissue O_2 consumption (Green and Stoner, 1950), might be expected to diminish the mitosis rate. The fall in the mitosis rate shown in the following experiments raises therefore many interesting questions concerning not only the remote control of mitosis, but also the nature of the alteration in carbohydrate metabolism produced by tissue injury.

METHODS.

The mice used were all Strong's CBA males of between 4 and 5 months of age. Since weaning they had been fed *ad libitum* on a mixed diet of maize, dog biscuit, rat cake soaked in cod liver oil, and chopped carrots, and they were all heavy mice in good condition.

The control mice were untreated, whilst the experimental mice were divided into two groups in which shock was induced by two different methods. One was the application, under ether anaesthesia, of tourniquets (6 turns of a size 25 rubber band) to both hind limbs for a period of 2 hours. The air temperature, an important factor in such experiments (Green and Stoner, 1950), was 18° C. The shock state very gradually develops after release of the tourniquets. The other method was the intraperitoneal injection of suitable doses of the Mg salt of adenosine triphosphate (ATP). The latter method of inducing shock, and the nature of the response, has been fully described (Green, 1943; Green and Stoner, 1950). The shock state begins to develop immediately following the injection, though it is not clinically very obvious in the first hour with the dosage used.

The mitosis rate was measured in the ear epidermis by means of the earclip technique (Bullough, 1948). Small pieces of ear were removed at intervals, fixed in Bouin's alcoholic fluid, embedded in wax, and cut into sections 7μ thick. All stages of mitosis were counted in unit section lengths of 1 cm. of the ear epidermis, and from each earclip 10 such counts were made and the average taken. From

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the averages obtained from each group of earclips, a grand mean and standard error was then calculated according to the method recommended for small samples by Simpson and Roe (1939).

RESULTS.

In the first experiment three groups, each of 5 male mice, were examined. Mice in the first group were the untreated controls, in the second tourniquet shock was induced, while in the third the animals were injected with 6 mg./10 g. body wt. MgATP. In these mice, and under the environmental conditions prevailing (Green and Stoner, 1950), this dose was shock-inducing, but not uniformly lethal. In the second group elastic bands were applied to both hind legs at 09.30 hr. and were removed at 11.30 hr. This period of vascular occlusion usually results ultimately in fatal shock. In the third group the injections were given at 11.30 hr.

To assess the changes in the mitosis rate, earclips were taken at 09.00 hr. and at 1½ hr. intervals until 18,00 hr. The results obtained are shown in Table I and Fig. 1.

TABLE I.—*The Changes in the Epidermal Mitosis Rate of Control Mice and of Mice in which Ischaemic Shock was Induced after the Removal of Tourniquets from the Hind Limbs or by Single Doses of MgATP (6 mg./10 g. body wt.) at 11.30 hr.*

Time of day.	Average numbers of mitoses per cm. length of sections cut 7 μ thick.		
	In 5 untreated control mice.	In 5 mice after removal of limb tourniquets.	In 5 mice after injection of MgATP.
09·00	2·2 ± 0·10	2·6 ± 0·20	2·6 ± 0·23
10·30	3·1 ± 0·22	2·7 ± 0·29	3·3 ± 0·30
12·00	5·2 ± 0·41	1·4 ± 0·11	2·5 ± 0·47
13·30	7·3 ± 0·12	0	2·5 ± 0·47
15·00	4·4 ± 0·12	0	2·9 ± 0·21
16·30	1·8 ± 0·14	0	1·2 ± 0·11
18·00	1·3 ± 0·23	0	1·0 ± 0·16

It is immediately obvious that the experimental conditions caused a marked depression of mitotic activity. In the control mice the mitosis rate increased to a maximum at about 13·30 hr., and previous work (Bullough, 1948) has established that this is a normal phenomenon associated with the afternoon sleep period. Thus the fluctuations observed in the mitosis rate of the control mice are merely part of the normal diurnal mitosis cycle.

In the experimental mice release of the tourniquets at 11.30 hr. was followed by a mitosis depression which completely eliminated the normal early afternoon mitosis wave. A similar, but not so pronounced, reaction was seen in the mice following injection with MgATP. Following the initial depression, the injected mice actually showed some increase in mitotic activity about 15.00 hr., although this was slight and transient. This difference between the two experimental groups was unexpected, since at the time of the experiment the injected mice showed a more severe degree of shock than the post-ischaemic group. The ATP-treated mice rapidly developed a fairly severe degree of shock, and at the end of

the experiment one of them died, though the remainder survived overnight. Tourniquet shock develops more gradually, and during the period of the experiment the mice were only mildly subdued, but after the end of the experiment they too, went gradually into a state of deep shock and during the following night all of them died.

An interesting point arises from the results obtained from the mice in tourniquet shock. At the time of the removal of the ligatures the epidermal mitoses were almost all in prophase and metaphase, since at this time the early afternoon

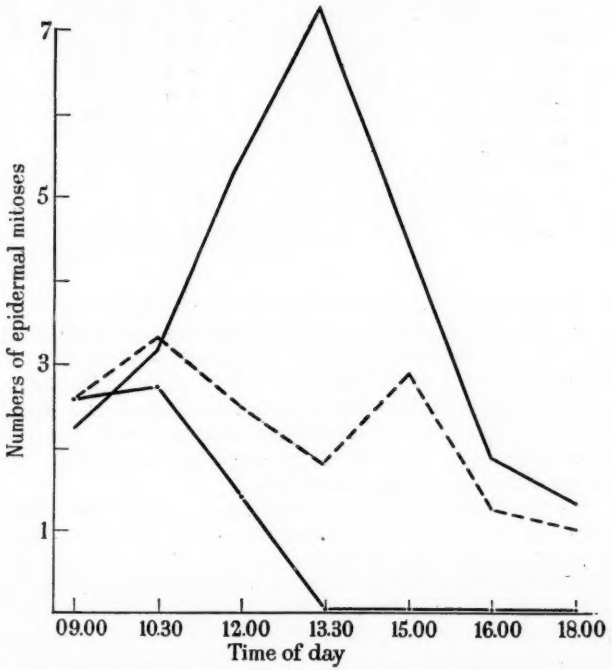

FIG. 1.—The changes in mitotic activity of the ear epidermis following the development of ischaemic shock. ——— Control ; - - - - - MgATP ; —.— tourniquet.

wave of mitosis was beginning. It was discovered that the conditions of shock did not prevent the completion of these divisions, and indeed this completion was evidently accomplished rapidly. Thus the effect of the shock was not to prevent the completion of a division, but only to prevent any more resting cells from entering the prophase.

In order to obtain a clearer understanding of this point, a second experiment was performed in which the earclips were taken at shorter intervals after the removal of the tourniquets. The first clip was taken at 10.00 hr. when the tourniquets were applied, the second at 12.00 hr., when they were removed, and further clips were taken at 15 min. intervals until 14.00 hr. The results obtained are given in Table II.

TABLE II.—*Average Numbers of Mitoses per Unit Length (1 cm.) of Sections of Ear Epidermis (cut 7μ thick) from Control Mice and from Mice which were Shocked at 12.00 hr. by the Removal of Tourniquets from Both Hind Legs.*

Time of day.	Control mice.		Shocked mice.	
	Numbers of mitoses.	Phases of division.	Numbers of mitoses.	Phases of division.
10·00	2·1 ± 0·17	. . . T	2·3 ± 0·12	. . . T
12·00	3·7 ± 0·20	P M . .	2·6 ± 0·16	P M . .
12·15	4·1 ± 0·15	P M . .	2·4 ± 0·18	P M . .
12·30	4·6 ± 0·24	P M . .	2·1 ± 0·25	. M . .
12·45	5·0 ± 0·31	P M A .	2·4 ± 0·16	. M A .
13·00	5·6 ± 0·26	P M A T	2·4 ± 0·19	. M A T
13·15	5·2 ± 0·29	P M A T	2·2 ± 0·20	. . A T
13·30	4·5 ± 0·21	. M A T	1·6 ± 0·14	. . . T
13·45	4·1 ± 0·15	. M A T	0·7 ± 0·11	. . . T
14·00	3·6 ± 0·18	. . A T	0	. . . .

In the phases of division, P represents the prophase, M the metaphase, A the anaphase and T the telophase.

It will be seen that the mitotic activity in the experimental mice fell rapidly to zero after the removal of the tourniquets at 12.00 hr. In the control mice, on the other hand, the mitosis rate rose to a maximum at about 13.00 hr., a phenomenon associated with the early afternoon sleep period (Bullough, 1948). In normal circumstances the mitotic activity remains high and a maximum is not reached until about 14.00 hr. That this did not happen in the present control series was almost certainly due to the interference with sleep caused by the removal of pieces of ear at 15-min. intervals.

However, the most interesting and significant part of the observations concerns the manner in which the mitotic activity fell in the shocked mice after the release of the tourniquets. At 12.00 hr. the rate of cell-division was increasing in both the control and the experimental mice, so that the mitoses counted were all in the pro- and metaphases. By 12.30 hr. no prophases remained in the shocked mice, and it is evident that no more developed. However, it appears that those mitoses which were already in existence at 12.00 hr. were completed at about the normal speed, the last of them finishing about $1\frac{3}{4}$ hours after the removal of the tourniquets. In contrast, the control mice continued to develop new mitoses until 13·15 hr., and, but for the disturbance of the experiment, would probably have continued to do so for some further time.

Haist and Hamilton (1944) showed that after the introduction of glucose into the stomachs of shocked rats there was a very large and sustained rise in blood glucose to levels much higher than in the controls. The already very low liver glycogen content was not increased by the glucose treatment. Since the injection of starch or glucose (Bullough, 1949*b*) causes a steep rise in the afternoon mitosis rate, it was of great interest to determine the effect of injected glucose on the skin mitosis rate in the shocked mouse. The details of such an experiment are given in Table III. In the footnote to this table the 1-hr. blood sugar figures of two separate small groups of mice treated at similar times to the main group are given, together with the skin mitosis rate at the time. The steep rise in the mitosis

TABLE III.—*The Changes in the Epidermal Mitosis Rate of Mice Injected Subcutaneously with* 20 *mg. Glucose at* 11.30 *hr.*

Time of day.	Average numbers of mitoses per cm. length of sections cut 7μ thick. In 5 normal mice.	In 5 mice shocked by removal of tourniquets at 11.30 hr.
09·00	3·8 ± 0·38	3·5 ± 0·55
10·30	2·1 ± 0·24	1·5 ± 0·19
12·00	3·8 ± 0·50	2·0 ± 0·24
13·30	12·7 ± 0·97	0
15·00	7·6 ± 0·57	0
16·30	4·1 ± 0·24	0
18·00	2·1 ± 0·18	0

Other mice :

Controls injected 20 mg. glucose at 11.30 hr. and killed at 12.30 hr. for blood sugar :

		Blood glucose. mg./100 ml.
Mouse No. 96—2·4 mitoses ,, ,, 97—3·8 ,,	M = 3·1	209 165

Shocked mice injected 20 mg. glucose at 11.30 hr. and killed at 12.30 hr. for blood sugar :

Mouse No. 93—0·8 mitoses ,, ,, 94—1·0 ,, ,, ,, 95—0·9 ,,	M = 0·9	270 298 249

rate of the normal mice following glucose injection compares strikingly with the rapid fall to zero in the tourniquet group. This in spite of the fact that the blood sugar is greater in the shocked group, and this rise (as other experiments have shown) is much further increased and then maintained during the time covered by this experiment, whilst the level in the normal mice is falling rapidly. The complete inhibition of mitosis is just as dramatic in the glucose-treated shocked mice as seen in the preceding groups, so that there can be little doubt that the cell is now refractory to the stimulus of glucose, or that the source of energy it obtains from glucose oxidation is not now available.

DISCUSSION.

The present experiments demonstrate clearly that a degree of tissue injury sufficient to induce a state of shock has a more powerful effect in depressing mitotic activity than any other factor yet discovered. It is already known that both insulin and phloridzin depress mitotic activity severely (Bullough, 1949*b*), and a similar effect is induced by starvation (Bullough, 1949c) and cold (Bullough, (1949*a*), but none of these cause such a complete stoppage of cell division as does the post-ischaemic state within a few minutes after the removal of the tourniquets.

This strongly suggests that a metabolite from the ischaemic limb is responsible for the antimitotic effect. A nervous mechanism is *a priori* unlikely, and in any case afferent reflex activity would be expected to be most pronounced at the time of application of the tourniquets rather than after their release. It is now known that the glucose or glycogen level of the tissues is of critical importance in the process of mitosis (Bullough, 1949*b*), and that shock or shock-inducing stimuli strongly disturb carbohydrate metabolism. The exact nature of this disturbance is not yet clear, and is being investigated on a large scale by the Sheffield Traumatic Shock Team. It is established that in ischaemic shock (Haist and Hamilton, 1944) liver glycogen falls almost to zero, and a similar process is seen in the mouse in both ATP and ischaemic shock (Green and Stoner, 1950). We have also recently found in the mouse that the skin glycogen content falls in ATP shock. However, it has to be remembered that it may take 4 hours or more to complete this glycogen discharge from the liver, and in the skin there was an apparent rise at 2 hr. with a moderate fall at 4 hr. We have observed in man, rabbit, rat and mouse that the blood sugar often rises steeply in the post-ischaemic state or after the injection of ATP, and in fact is usually already considerably elevated before the tourniquets are released, due presumably to adrenaline discharge. The raised blood sugar can be reduced by insulin, and the liver glycogen rises—the capacity to synthesize glycogen is not truly lacking. It may be that the complete stoppage of mitosis is related in some way to a failure of glycogen synthesis, though it is obviously not due directly to glycogen deficiency nor to lack of glucose, of which the tissues have an excess supply. The effect may be due to an interference in the tricarboxylic acid cycle of carbohydrate metabolism, but it would be a little premature to speculate further.

The nature of the interfering agent is not known, but it presumably comes from the post-ischaemic limb, and the same process is set in motion by the injection of shock-inducing doses of ATP. Incidentally, this nucleotide inhibition can be just as pronounced as that seen in the post-ischaemic state. The process occurs so quickly that it is not likely to be merely a non-specific expression of the generally diminished bodily activities of the shock state. It occurs long before the fall in temperature, diminished O_2 consumption, etc., typical of the shock state are pronounced. It is more likely to be due to a direct chemical, or possibly an indirect hormonal effect, set in motion by the ischaemic metabolite(s). If a hormonal effect, attention would be naturally directed to the anterior pituitary and the suprarenal gland in view of their many important effects on carbohydrate metabolism. There is some evidence suggesting that under similar conditions, even with very small stimuli of this type, pituitary antidiuretic hormone is released (Green and Stoner, 1950). Vogt (1949) finds that, when present in the perfusing fluid, ATP and K, both possible metabolites from an ischaemic tissue, increase the output of cortical hormones, as assayed on adrenalectomized rats. There is also the work of Selye (1946) demonstrating an increased output of cortical hormones in the stage of " counter-shock " following stress and alarm stimuli. Although at this stage speculative, the changes in carbohydrate metabolism set in motion at the very beginning of the shock state are sufficient to suggest that the metabolites from an ischaemic limb stimulate the output not only of adrenaline but of adrenal cortical hormones, possibly indirectly through the pituitary gland. If this is so, then the gluco-corticoids may prove to be the active agents in the antimitotic effect, a point that is to be experimentally tested.

Although of the highest importance, we are not primarily concerned in this paper with the nature of the metabolite(s) from ischaemic tissue which mediates this powerful effect on mitosis and carbohydrate utilization. Obviously the role of ATP and other nucleotides must be investigated in this connection, since it has a precisely similar action. The difficulty is in deciding whether ATP acts directly and specifically in the effects described, or whether they are merely indirect reflections of its shock-inducing action. Present evidence favours the former view, but for the present the issue must be left there.

Another point of fundamental interest is the way in which the depression of mitosis develops. The effect is felt at only one point in the process of mitosis, that of the change from the resting cell to the prophase. All those mitoses which were already in existence at the time of the release of the tourniquets were able to complete their divisions and to reach the resting phase once more. But the development of any more prophases was inhibited altogether. This phenomenon has been seen before, particularly in the insulin experiments, but it has never been seen so clearly.

From these observations the important conclusion emerges that it is the initial stage of a division which is the most susceptible to glycogen shortage ; and that it is only in the initial stage that significant quantities of glucose are consumed. If, as has been suggested, the importance of available sugar in mitosis is for the production of energy, then it is the change form the resting phase to the prophase in which this energy is mainly consumed.

Many attempts have been made to measure the energy requirements of dividing cells by determining changes in the respiration rate. This work has been done mainly with dividing eggs, particularly of sea urchins and frogs, and because of the difficulty of the technique there has been much disagreement over results. However, it is interesting to mention here that two of the most reliable sets of results, those of Runnström (1933) and Brachet (1934), agree that the greatest rise in oxygen consumption and carbon dioxide output coincide with early prophase. Thereafter the respiration rate remains low until it rises again at the time of laying down of the new cell wall. However, the most recent work of Zeuthen (1946) has indicated that there is no significant oxygen uptake associated with cytoplasmic cleavage, although in other respects his results coincide with those of Runnström and Brachet.

The results we have recorded thus draw together in an interesting way the nature of the energy changes involved in mitosis, and the physiological mechanism by which natural antimitotic agents may work. They also serve as another pointer in favour of the hypothesis, previously developed from other data, that the adenine nucleotides induce shock by interfering at some stage in the metabolic cycle of the carbohydrates.

SUMMARY.

In the initial stages of the general reaction to injury (shock) following the release of hind-limb tourniquets or shortly following the injection of shock-inducing doses of adenosine triphosphate, mitotic activity in the skin of mice falls sharply or ceases entirely.

This mitotic inhibition, which lasts at least 6½ hours, occurs in the presence of a high tissue glucose level, which in normal mice is associated with a steep rise in the mitotic rate.

The effect is felt only at the change from the resting cell to the prophase, and mitoses already in existence complete their division, suggesting that it is only in the initial stage of mitosis that significant quantities of glucose are consumed.

Some facts are given in support of the hypothesis that metabolites from the post-ischaemic limb and also injected ATP interfere with carbohydrate utilization, possibly indirectly by a hormonal mechanism, and thus prevent mitosis.

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ASCORBIC ACID AND THE PRODUCTION OF ANTIBODY IN THE GUINEA-PIG.

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In discussing work on the relation of vitamins to immunity, Watson (1937) noted that "the voluminous literature, and specially such reviews as those of Clausen (1934) and Robertson (1934), reveal a mass of confusing and contradictory statements based on experiments carried out on an inadequate scale, and without regard to simple statistical requirements. The evidence, taken as a whole, suggests that Vitamins B and D have little, if any, influence on resistance, while in the case of Vitamin C the observations are particularly confusing and difficult to interpret." Perla and Marmorsten (1937) concluded that Vitamin C had no relation to the production of antibodies, either naturally or in response to active immunization.

At present ascorbic acid is generally considered to have but two uses, the prevention and treatment of scurvy (Pijoan and Lozner, 1944; Schneider, 1946). Wilson and Miles (1946) state: "If we attempt to assess the significance of the data at present available with regard to the influence of variations in vitamin intake on resistance to infection, we shall probably conclude that the evidence with regard to the effect of Vitamin C deficiency is so divergent that no sound conclusion is possible at the moment, though there are definite suggestions that animals such as the guinea-pigs, that are peculiarly susceptible to deprivation of the vitamin, are rendered more susceptible to infection when it is withheld."

Much of the confusion arises from failure to recognize the importance of the species of experimental animal. Vitamin C deficiency can be studied only in animals, which, like the guinea-pigs and the primates, are dependent on their diet for ascorbic acid, and which develop symptoms of deficiency if the vitamin is withheld. Withholding ascorbic acid from the diet of an animal which can synthesize Vitamin C does not make the animal deficient. Such animals can be used to study the effects of an excess, but not of a deficiency. Further confusion may arise unless a distinction is made between the response following a single injection of antigen (e.g. the primary response in the case of diphtheria toxoid) and the response to antigen of an animal that has already received one or more doses (the secondary response). Measurements of primary response, unless carefully made, are unlikely to yield significant results. The blood antibody response to a single dose of antigen is small, relatively slow in developing, rises and falls rapidly, and is less affected by the dose of antigen than the secondary response. The blood antibody response to more than one injection of antigen is large, develops quickly, rises rapidly, is maintained for a relatively long time,

and its magnitude depends largely on the dose of the antigen. It follows that real differences in primary responses tend to be small, and are in evidence for only a short period of time, and can be detected only by precise methods of estimating antibody. On the other hand, differences in the secondary response tend to be large, and persist for long periods, and can be detected by comparatively crude methods of estimating antibody.

With these considerations in mind, the effect of ascorbic acid deficiency on the primary and secondary antibody response of guinea-pigs to diphtheria toxoid was measured.

EXPERIMENTAL.

Three groups, each of 15 albino guinea-pigs of approximately 350 g. in weight, were given a dry, pelleted diet, containing very little ascorbic acid, but complete

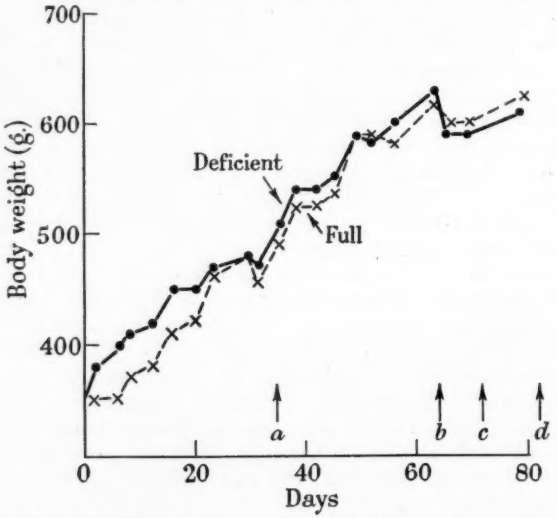

FIG. 1.—Changes in body weights of guinea-pigs given full diet and Vitamin C deficient diet. The arrows indicate the date of—
(*a*) primary toxoid stimulus;
(*b*) intradermal tests of primary response;
(*c*) secondary toxoid stimulus;
(*d*) heart puncture for measurement of secondary response.

in all other respects (Bruce and Parkes, 1947). One group was given 5 mg. of ascorbic acid three times a week. To avoid oxidation of the vitamin, all solutions were made up freshly, and administered without delay by stomach-tube. The second group was given unlimited cabbage (Hartley, 1943) as a natural source of Vitamin C. The animals in all three groups gained weight at approximately the same rate. Fig. 1 records the weights of the vitamin excess and vitamin deficient groups. These results suggest that the vitamin deprivation had not produced a grossly deficient group of animals. Indeed, Bruce and Parkes (1947) believe that small, though slightly variable quantities of ascorbic acid are present in the diet. Post-mortem examination at the end of the experiment

confirmed this impression. There was no macroscopic evidence of deficiency, and none of delayed healing in the lesions made by the intradermal injection of diphtheria toxin used in the test of immunity.

On the thirty-fifth day all animals were immunized by the subcutaneous injection of 0·5 Lf of alum-precipitated diphtheria toxoid in 1 ml. of normal saline. On the sixty-third day a rough indication of the degree of ascorbic acid saturation of each group of animals was obtained by Rötter's (1938) test, which depends on the rate of decolorization of the redox dye 2:6-dichlorophenolindophenol injected intracutaneously. The solution of the dye, syringe, needle, site and method of injection were the same throughout, and there was

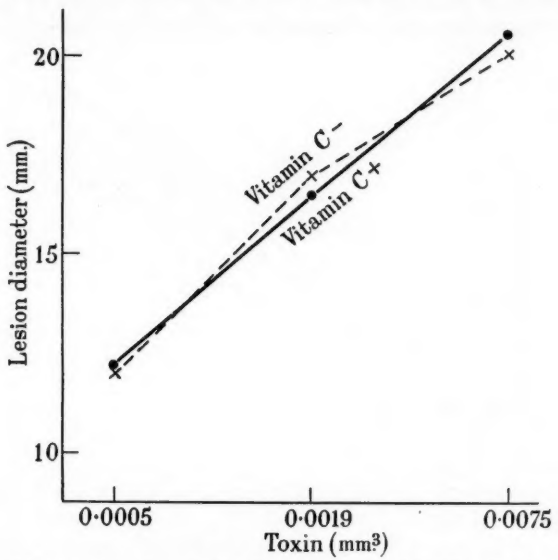

FIG. 2.—Susceptibility to intradermal diphtheria toxin of skin of guinea-pigs receiving full diet and Vitamin C deficient diet.

within groups close agreement of the times taken by individual animals to decolorize the dye. The difference between groups was striking. Animals given ascorbic acid took an average of two minutes, and those given cabbage five minutes, to decolorize the dye. In the deficient group the colour persisted for more than 30 minutes.

Primary response.—On the same day the primary response was measured by the intradermal injection of graded doses of diphtheria toxin. The response obtained when the inflammatory lesion-diameter at 24 hours is plotted against the log dose of toxin in passively-immunized guinea-pigs is linear; different dose-response curves are substantially parallel, and the shift in the dose-response curve is proportional to the antitoxin content of the blood (Miles, 1949). By fitting regression lines to the dose response curves so obtained, it is possible to detect relatively small, but significant differences, in the antitoxic immunity in different groups of animals. Subsequent tests made in this laboratory showed

the same relations held in actively-immunized animals (*cf.* Hartley, 1934). If skin reactivity to toxin were to be valid as a measure of antitoxic immunity, it was necessary to show that sensitivity to diphtheria toxin was not altered by ascorbic acid deficiency alone. Two groups of guinea-pigs were given the pellet diet, and in addition one group was given the 5 mg. of ascorbic acid three times a week, for 30 days. The skin reactivity tested by the intradermal injection of graded doses of diphtheria toxin was substantially the same in both groups (Fig. 2).

The three groups of immunized animals were accordingly tested by graded doses of intradermal toxin. Fig. 3 shows the results ; each point on the graphs represents the mean of readings from 15 animals. The results of this experiment were subjected to an analysis of variance (Table I). There is no significant

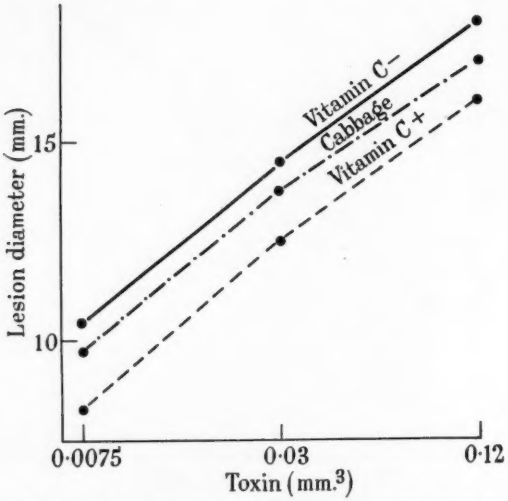

FIG. 3.—The response of guinea-pigs on various diets immunized by one injection of toxoid to graded doses of intradermal diphtheria toxin.

TABLE I.—*The Analysis of Variance of the Data of the Experiment Recorded in Fig.* 3.

	d.f.	Mean sq.	F.	P.
Between groups	2	47·5908	1·53	>0·05
,, doses	2	609·1685	19·55	<0·001
Linearity	1	1213·6694	38·95	<0·001
Departures	1	4·6676	0·15	>0·05
Dose/guinea-pig interaction	84	1·3646	0·04	>0·05
Group/dose interaction	4	0·5102	0·02	>0·05
Between animals within groups (error)	42	31·1582		
Total	134			

Slope = 6·09943.

difference between the three groups of animals. It should be noted, however, that the error term in the analysis is the variance between animals within groups, that there are large differences in the reactions of different animals. In addition, the variance ratios for the interactions are remarkably small, and this may also indicate a relatively large error term. It seems probable, indeed, that the differences between the three groups, which are comparatively regular (Fig. 3), are in fact real though small, and that an experiment with larger numbers of animals in each group would have produced statistically significant differences. It is noteworthy that if the method had not been amenable to statistical analysis

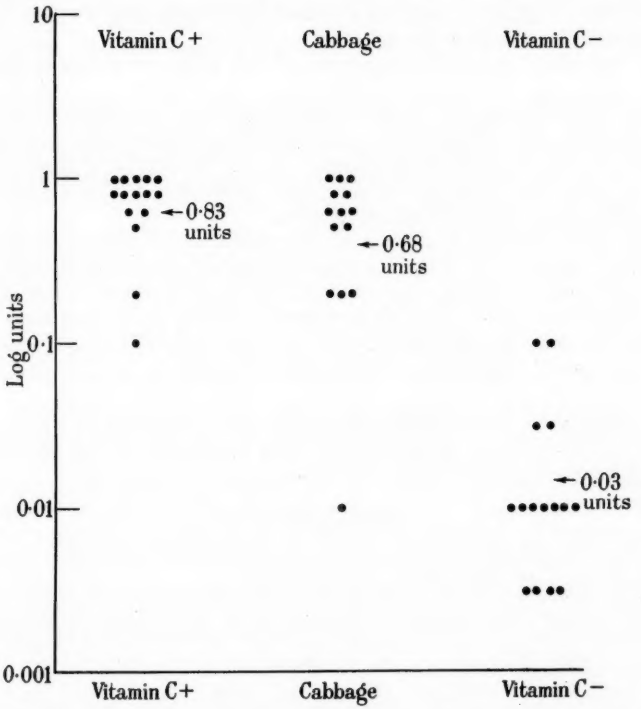

FIG. 4.—Serum antitoxin levels in guinea-pigs on various diets in response to a secondary stimulus of diphtheria toxoid.

and had been judged by an impression of the minimal reacting dose, as in the classical multiple Schick dose method (Glenny, Hopkins and Pope, 1924), a significant effect would have been deduced.

Secondary response.—The animals were given a second dose of antigen seven days after the intradermal injection of toxin. Ten days later the antitoxin content of the sera was estimated by the intradermal method (Römer and Sames, 1909).

The blood antitoxin clearly measures the secondary response, both to the

test toxin given intradermally and to the alum-precipitated diphtheria toxoid injected subcutaneously. The mean titre (Fig. 4) was very low in the ascorbic acid deficient animals, being about one-thirtieth of that in the controls; and animals given ascorbic acid had slightly more circulating antitoxin than animals receiving cabbage.

CONCLUSION.

In comparison with guinea-pigs receiving an excess of ascorbic acid—

(*a*) The primary antitoxin response of ascorbic acid deficient guinea-pigs to alum precipitated diphtheria toxoid was not significantly reduced, though it is probable that if larger groups of animals had been used in the experiment a slight reduction would have been shown.

(*b*) The secondary antitoxin response of the deficient animals was greatly reduced.

Since the indicating effect in the measurements of antibody is the neutralization of toxicity, the difference in antitoxin levels cannot be attributed to a modification of the serological (as distinct from the immunological) properties of circulating antibody as a result of vitamin deficiency; and almost certainly reflects a difference in the state of immunity.

It is clear that Vitamin C cannot be dismissed as having no direct influence on the immune state. The nature of the influence is obscure, though the comparatively small effect of deficiency on the primary response suggests that the vitamin is concerned more with the metabolic systems governing the production of antibodies in cells already in a state of "secondary responsiveness" than with the response of those cells to primary conditioning by the antigen.

It is a pleasure to acknowledge the advice and criticism of Dr. A. A. Miles, and helpful information on diets from Miss H. M. Bruce. I am indebted to Dr. W. L. M. Perry for the statistical analysis.

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THE CHEMOTHERAPY OF EXPERIMENTAL TUBERCULOSIS IN MICE.

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THE use of mice in the experimental chemotherapy of tuberculosis has been described by Martin (1946), Raleigh and Youmans (1948*a* and *b*), and Rake, Jambor, McKee, Pansy, Wiselogle and Donovick (1949); Martin, and Rake *et al.* estimated the activity of chemotherapeutic agents by their power to prolong the survival-time (expressed either as arithmetic mean, or 50 per cent mortality-time) of mice in treated groups. Raleigh and Youmans formulated a more elaborate system based upon histopathological assessment of the lesions.

The pathogenesis of experimental tuberculosis in mice was studied in detail by Raleigh and Youmans (1948*a*). These workers used a standard inoculum of 0·1 mg. *M. tuberculosis* H.37.RV. and described two types of pulmonary lesion; a "necrotic-exudative" lesion, affecting 80 per cent of their mice, and a proliferative lesion affecting 20 per cent. Stewart (1950) noted that the type of lesion found in experimental tuberculosis in mice was determined largely by the size of inoculum and virulence of the infecting bacillus. Inoculation of mice with 0·75 mg. *M. tuberculosis* (human) Strain 905 caused lesions which were uniformly necrotic and rapidly lethal. Smaller inocula, or less virulent organisms, gave rise to proliferative lesions and longer survival, though the infection remained progressive in its course. A simple method of pathological assessment of standard experimental infections, based upon these findings, was devised.

In the present report an attempt has been made to correlate data obtained from pathological assessment on one hand, and survival-time on the other, in groups of infected mice treated with known anti-tuberculous agents.

METHODS.

Stock cultures.

The organism used throughout these studies has been a "human" culture of *Mycobacterium tuberculosis*, Strain 905, maintained as described by Martin (1946).

Preparation of suspensions.

Suspensions were prepared by milling in a bottle containing small steel balls a weighed amount of growth removed from a 17-day-old culture on Lowenstein's medium. The organisms were milled without added water for 90 seconds, then 1 ml. of distilled water was added and milling was contained for a further 90

seconds. Finally, the amount of water necessary to give a suspension containing 7·5 mg. per ml. was added and milling was continued for a third period of 90 seconds. After removal from the bottle this suspension was diluted further with distilled water if required. All inocula were given intravenously in a volume of 0·1 ml. For the " acute " infection the inoculum was 0·75 mg., and for the " chronic " infection 0·02 mg. (moist weight).

Establishment of experimental groups.

Male white mice were used in the experiments. All the mice weighed between 18 and 22 g. at the time of infection. The total number of mice required for the experiment, whose weights were within this range, and who had been born within a week of one another, were drawn from stock. They were then separated into groups weighing 18–19 g., 19–20 g., and so on. Taking each of these weight groups in turn, two mice at a time were allocated to each of the planned experimental groups. When all these had been allocated the process was repeated with each of the other weight groups in turn. In this way not only is the average weight of the mice in each experimental group found to be the same, but the distribution of individual weights within each group is approximately the same.

The following groups were set up for the main experiments described below:

Two groups of untreated controls, each of 20 mice.

Two groups treated with *p*-aminosalicylic acid (P.A.S.), one of 20 and one of 35 mice. The drug was given by admixture with the food to the extent of 1 per cent, thus ensuring an intake of about 40 mg. of drug per 20 g. mouse per day.

Two groups treated with streptomycin, one of 20 and one of 50 mice. The drug was given subcutaneously (1000 units per 20 g. mouse), at approximately 9 a.m. and 4 p.m. from Mondays to Fridays.

Two groups treated with *p*-ethylsulphonylbenzaldehyde thiosemicarbazone (No. 8388), one of 20 and one of 50 mice. The drug was given by admixture with the food to the extent of 0·25 per cent., thus ensuring an intake of about 10 mg. of drug per 20 g. mouse per day.

The smaller of each of the above pairs of groups was set aside for the collection of data on weight changes and mortality; mice from the larger group of each pair were sacrificed at intervals to provide histological data.

In two sets of experiments described in this paper the drugs were given as follows:

(1) Continuously from the day of inoculation until death.
(2) For the first 14 days after inoculation.

In the third experiment groups were arranged on the same general lines, but *p*-amino-salicylic acid was not given. Treatment with streptomycin or 8388 was begun on the 21st day after inoculation and continued until death.

In all experiments of this type, where large numbers of mice have been inoculated intravenously, special steps have been taken to reduce the importance of any changes which might occur during the time taken to complete the inoculations. Such changes might occur in the bacterial suspension (although none has been detected in suspensions kept for a much longer time than is taken to complete the inoculation of all the mice in one experiment), in the accuracy of

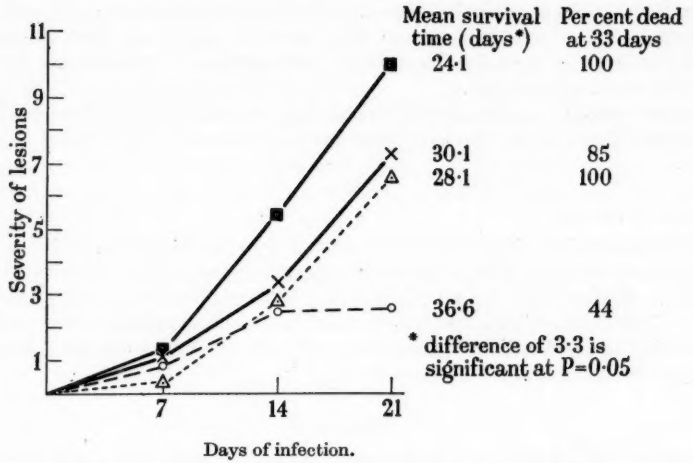

FIG. 1.—Effect of 14 days' treatment in mice infected with 0·75 mg. *M. tuberculosis*.

■——■ Untreated. ○- - -○ 8388.
×——× Streptomycin. △....△ *p*-aminosalicylic acid.

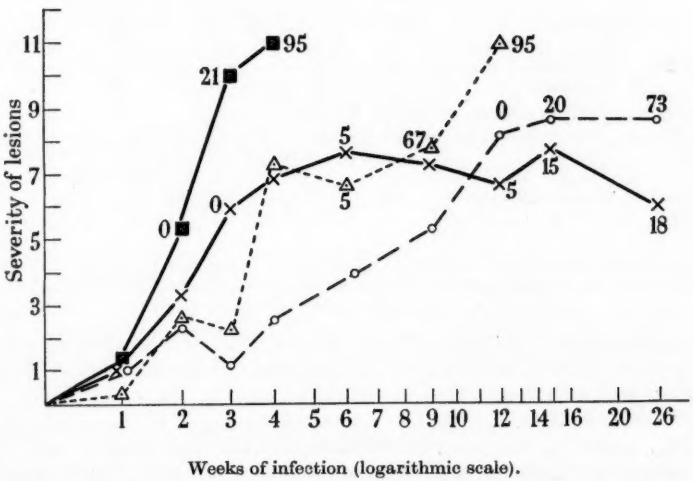

FIG. 2.—Effect of continued treatment in mice infected with 0·75 mg. *M. tuberculosis*. The figures on the graphs refer to the per cent mortality in the groups.

■——■ Untreated. ○- - -○ 8388.
×——× Streptomycin. △....△ *p*-aminosalicylic acid.

manipulation by the operator and possibly in other, unknown circumstances. The mice in any one experimental group are divided into three equal sub-groups, in separate cages. One-third of the mice in each group are then infected first, followed by the mice in each of the second sub-groups, and finally by the mice in each of the third sub-groups.

All mice were fed on powdered food of the composition described by Thomson (1936), containing 14 per cent of dried milk. Water was available to them at all times.

Assessment of results.

The progress of the infection was assessed by recording all deaths and noting the presence or absence of specific lesions post-mortem. The mice were weighed each week. Tissues were taken for pathological examination from mice, sampled at random in batches of at least three from each group, at stated intervals throughout the infection. Details of the system of pathological assessment are given in a previous communication (Stewart, 1950).

Results.

The effects of 14-day and continued treatment with the substances named upon the 0·75 mg. " acute " infection are shown in Fig. 1 and 2. The effect of delayed treatment (begun at 21 days and continued until death) upon the 0·02 mg. " chronic " infection is shown in Table I.

Table I.—*Effect of Streptomycin and* 8388 *in Mice Infected with* 0·02 *mg.* M. tuberculosis. *Treatment was begun* 21 *days after Infection.*

Day of infection.		21.	28.	35.	49.	63.	70.
Controls untreated	Per cent mortality	—	11	38	48	60	63
	Mean weight in g.	30	29·2	30·5	32	..	..
	Lesions*	..	10·2	9·4	8·9	..	..
Streptomycin 1000 units twice daily	Per cent mortality	—	—	—	—	—	—
	Mean weight in g.	30	30	32	34	37	37·5
	Lesions	..	7·3	7·8	8·4	..	..
8388 0·25 per cent in food (10 mg. per day)	Per cent mortality	—	—	2	11	22	26
	Mean weight in g.	30	25·5	27	31·5	32·5	32
	Lesions	..	9·3	8·6	8·2	..	..

* The severity of the lesions was assessed in terms of the gross and microscopic changes, and number of bacteria, in the lung by the method described in a previous paper (Stewart, 1950).

It is evident that, in terms of final results, the survival-time of mice in the various groups reflects accurately the progress of the disease as assessed by pathological examination. This is clearly shown in the experiment involving 14 days' treatment (Fig. 1). In the intermediate stages of the prolonged experiments (Fig. 2 and 3, Table I), correlation between pathological and mortality data is less exact. Thus, on the 60th day of one experiment (Fig. 2), mice treated with PAS suffered 67 per cent mortality, whereas mice treated with streptomycin showed only 5 per cent mortality, although in both groups the lesions appeared to be equally advanced; furthermore, all the mice in the P.A.S. group died by the 84th day, while 80 per cent of the streptomycin-treated mice survived for more than 182 days. Weight graphs (Fig. 3) showed that the mice in the P.A.S.

group lost weight from the 30th day of infection, whereas those in the other groups gained steadily. Other experiments showed that 8388 depressed appetite, and caused some loss of weight in the early days of treatment; this effect was temporary but, since the compound was given in the food, it obviously caused a lowering of the daily intake of 8388.

The relatively high pathological scores of the streptomycin-treated mice, early in the infection, were accounted for by a considerable multiplication of bacteria occurring between the 28th and 60th days; but the gain in weight and survival of the mice indicate that the drug was able to arrest the disease in spite of this multiplication.

These findings support the opinion of Raleigh and Youmans (1948*b*) that exact information on the chemotherapy of murine tuberculosis can only be gained from pathological studies at various stages of the infection. It is obvious that prolongation of survival-time gives a fair indication of the fact that a substance is active and, in general terms, represents the degree of suppression of the disease.

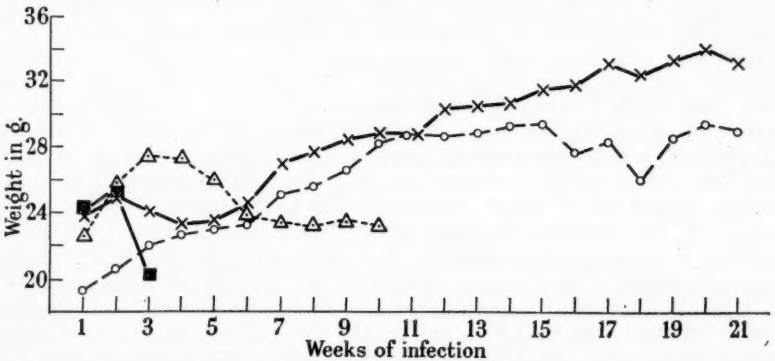

FIG. 3.—Weights of mice infected with 0·75 mg. *M. tuberculosis*. Effect of continued treatment. Each point represents the mean weight of a group of mice.

■——■ Untreated. ○- - -○ 8388.
×——× Streptomycin. △....△ *p*-aminosalicylic acid.

Survival-time does not, however, reveal the degree of development of sub-lethal lesions and, conversely, random pathological observations are not necessarily indicative of the ultimate state of the infection. Information on the mode of action of therapeutic agents therefore requires correlation of data from all three sources—weight, mortality and pathological assessment—applied to long-term experiments.

The efficiency of the chemotherapeutic agents tested.

The three substances used in these experiments are known antituberculous agents. Streptomycin and 8388 were each markedly superior to P.A.S., and appeared to be comparable to each other in therapeutic activity until the 105th day of continuous treatment (Fig. 2). Thereafter, mice treated with 8388 succumbed gradually to the infection. With delayed treatment the superiority of streptomycin was more clearly shown (Table I).

Tubercle bacilli were re-isolated from the lungs of infected mice in each of the treated groups after 80 days' treatment. After 10 days' growth on Lowenstein's medium, fresh mice were inoculated with each strain and treated with the appropriate substance, at the same dose-levels as in the original experiment. No significant differences in therapeutic response were observed, showing that, under the conditions described, tubercle bacilli failed to develop resistance to streptomycin, P.A.S. or 8388 *in vivo*.

Several apparent differences in the modes of activity of the substances are evident. Streptomycin and P.A.S. allowed the bacilli to multiply rapidly between the 21st and 60th days ; streptomycin then appeared able to arrest any further advance of the infection, though not to sterilize it, but P.A.S. was unable to inhibit the rapid development of lethal lesions. Given continuously, 8388 arrested the disease very effectively in the earlier stages, and this compound appeared more active than streptomycin at the 60th day ; thereafter, however, illness developed gradually, and this appeared to be due to specific lesions rather than to a cumulative toxic action of the compound. When treatment was delayed, 8388 was less effective than streptomycin.

DISCUSSION.

The anti-tuberculous action of thiosemicarbazone compounds was first described by Domagk, Behnisch, Mietzsch and Schmidt (1946), and Domagk (1947), from experimental studies. Clinical trials in cases of lupus, pulmonary tuberculosis and surgical tuberculosis were claimed to have shown promise (Moncorps and Kalkoff, 1947 ; Domagk, 1948 ; Kuhlmann, 1948). A critical summary of the extensive German clinical work with TbI/698 (the thiosemicarbazone of *p*-acetylaminobenzaldehyde) has been published by Hinshaw and McDermott (1950). The compound used in the present investigation, *p*-ethylsulphonylbenzaldehyde thiosemicarbazone (8388), is one of the most active members of the series under experimental conditions. In the exacting tests described above, 8388 displayed a promising degree of therapeutic efficacy, and appeared to approach streptomycin more closely than any other synthetic compound so far described. The German workers seem to favour the thiosemicarbazone derived from *p*-acetylaminobenzaldehyde.

The therapeutic response of mice treated with each substance was associated with more or less prolongation of the proliferative stage of tissue-reaction to the infection. It has already been shown that a similar effect obtains when mice are infected with less virulent, or smaller inocula of virulent tubercle bacilli (Stewart, 1950). As the dose of virulent bacilli is reduced, necrotic lesions become less common or altogether absent, and survival-time increases proportionately. It appears, therefore, that anti-tuberculous agents act in mice in a manner comparable to a reduction in the numbers or virulence of the infecting bacilli. That is, they reduce the inoculum to a quantity which the natural resistance of the mouse can antagonize. With streptomycin survival could be remarkably prolonged, but proliferative lesions containing acid-fast bacilli were still detected 211 days after infection, and withdrawal of the drug allowed the disease to become reactivated. Neither the resistance of the mouse nor the anti-bacterial action of the drug were sufficient to sterilize the infection, or even to cause a significant degree of remission.

These findings suggest that the maximum degree of activity that can be

expected among the range of known anti-tuberculous agents, tested in mice, is retardation of the disease. Evidence of regression or true healing can be sought only in certain other species of laboratory animals, or in man. With this reservation, murine tuberculosis provides a valuable means of screening and to some extent grading therapeutic agents. We venture to predict that a substance which could be shown to cause true regression of tuberculosis in mice would be of high promise for clinical use.

SUMMARY.

Streptomycin, *p*-ethylsulphonylbenzaldehyde thiosemicarbazone and *p*-aminosalicylic acid were compared in therapeutic tests against experimental tuberculosis in mice.

The thiosemicarbazone compound showed higher activity than *p*-aminosalicylic acid, and was superior to streptomycin in retarding the early stages of the disease. In the later stages streptomycin was more effective.

In the assessment of therapeutic activity the survival-time of treated mice reflected accurately the final state of the disease. Variations in the course of the disease and differences in the actions of the drugs could only be studied by correlating the growth, mortality and pathological changes in treated groups of mice.

Therapeutic activity in the compounds tested was comparable in effect to reducing the numbers or the virulence of the bacilli inoculated. True remission or sterilization of the infection did not occur.

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VARIATION IN LABORATORY STOCKS OF INFLUENZA VIRUSES: BIOLOGICAL CHARACTERS OF VARIANTS.

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THE occurrence of variation in viruses has long been recognized. It has been suggested, for example, that vaccinia virus originally arose as a variant of variola virus, that the 17D strain of yellow fever virus is an avirulent variant, and that the influenza pandemic of 1918–19 was caused by a highly virulent influenza virus variant. Variation in viruses has been extensively reviewed by Findlay (1939), and Burnet (in press) has recently given a detailed account of variation in influenza viruses. Laboratory study of virus mutants has been largely concerned with bacteriophages. These have been described by Sertic (1929), Burnet and Lush (1936), Gratia (1936), Luria (1945), Hershey (1946), Delbrück (1948), and Hershey and Rotman (1948).

Detailed study of mutations in influenza viruses has been restricted to the O–D mutation described by Burnet and Bull (1943) and Burnet and Stone (1945). It was shown that influenza A viruses exist in two forms: the O or original form agglutinates human or guinea-pig cells, but not fowl cells; the D or derived form agglutinates fowl cells to approximately the same titre as human cells. As isolated from human beings with influenza, the virus is in the O form. However, on cultivation of the virus in the chick embryo, a proportion of D mutants arises whose survival is selectively favoured. It was found that passage of O virus by the amniotic route at low dilution soon brought about an alteration in the character of the fluids to the D form. The virus could be maintained in the O form indefinitely by passage at high dilution, i.e. by using an inoculum which did not include any D virus.

In the course of studies on the properties of influenza virus produced in the first "burst" following initiation of infection (Henle, Henle and Rosenberg, 1947), we observed cultures of Melbourne virus which differed from normal Melbourne virus in their position in the fowl cell receptor gradient (Burnet, McCrea and Stone, 1946). It soon became clear that this difference was not due to any unusual properties of virus produced in the first burst, but to the presence of a variant in our laboratory culture. The variant was not observed when the stock MEL was passaged at low dilution, but only at limiting dilution, that is under conditions when infection might be induced by a single infective virus unit.

It was soon recognized that the occurrence of variants was not confined to this strain, but was found in other Institute laboratory strains. Two aspects

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of these findings have been of interest. First, one of the variants has shown alterations in virulence and multiplication which may be related to concurrent changes in other biological characters. Secondly, the presence of variants in virus cultures has some important implications in the laboratory cultivation of viruses. These aspects have been treated separately in this and the following paper.

MATERIALS AND METHODS.

Virus strains.

Influenza A : PR8—American strain.
MEL—Melbourne strain.
Ocean Island—1948 strain isolated from Ocean Island.
Influenza B : LEE—Classical strain.
Newcastle Disease Virus of Fowls (NDV) : Victorian strain of Albiston and Gorrie.

Horse-serum saline.

This contains 10 per cent by volume of normal horse serum in normal saline.

Fowl red cells.

Blood was obtained from the wing vein of adult white leghorn fowls. The cells were washed three times with normal saline and made up to a 1 per cent suspension in normal saline. A 1 per cent suspension of human red cells was prepared in the same way from human group O blood.

RDE.

An extract of the 4Z strain of *V. cholerae* containing the receptor-destroying enzyme (RDE) was prepared as described by Burnet and Stone (1947).

RDE-treated red cells.

Suitable dilutions of RDE were prepared in calcium-acetate saline (a sodium acetate-acetic acid buffer adjusted to pH 6·2 and containing 0·1 per cent calcium chloride). To 4 ml. volumes of each dilution, 0·1 ml. of packed fowl red cells was added and incubated for 30 minutes at 37° C Sodium citrate was then added to a final concentration of 2 per cent and the cells were centrifuged, washed in 2 per cent citrate in saline and finally suspended in sufficient 2 per cent citrate saline to make a 1 per cent red cell suspension.

Ovomucin.

Ovomucin was prepared from egg-white by the method described by Gottschalk and Lind (1949).

Antisera.

Antisera were prepared by bleeding ferrets 14 days after intra-nasal inoculation with 0·25 ml. of infected allantoic fluid diluted 1 in 10 in horse-serum saline.

Haemagglutination titration.

Serial twofold dilutions of virus (0·25 ml.) were prepared in normal saline and an equal volume of 1 per cent fowl red cells was added to each dilution.

The cells were allowed to settle at room temperature, and readings were made by observing the pattern of sedimented cells. Partial, one plus (+) agglutination was taken as the end-point, and the least amount of virus which will produce one plus agglutination is referred to as one agglutinating dose (A.D.).

Agglutination of RDE-treated cells.

Fowl red cells (0·25 ml. volumes) treated with falling concentrations of RDE were distributed in test tubes, and an equal volume of virus containing 5 agglutinating doses (A.D.) was added to each tube. The cells were allowed to settle at room temperature and readings were made by the pattern method.

Antihaemagglutinin titration.

Sera were inactivated shortly before use by diluting 1 in 5 in normal saline and heating at 62° C. for 15 minutes. Serial dilutions of the antisera (0·25 ml.) were prepared in normal saline and an equal volume of 1 per cent fowl cells was added. A third volume containing 5 A.D. of the test virus was added, mixed, and the cells allowed to settle at room temperature. Readings were made by the pattern method, and the least amount of antiserum causing one plus agglutination was taken as the end-point. The antihaemagglutinin titres are expressed as the reciprocal of the initial dilution of antiserum at the end-point.

Electrophoretic mobility.

The electrophoretic mobility of red cells was measured by microelectrophoresis (Ada and Stone, 1950*b*).

Heated virus.

As indicator virus in inhibitor titrations the following were used :

Heated LEE.—Allantoic fluid was heated at 56° C. for 30 minutes.

Heated MEL.—(Modified from Stone, 1949*a*.) To 6 parts of MEL allantoic fluid, 2 parts by volume of 2 per cent sodium citrate and 1 part of standard borate buffer, pH 8·5, were added. This was heated for 30 minutes at 56° C.

When heated MEL virus was required in interference experiments, it was diluted in the same way and heated at 56° C. for one hour.

Inhibitor destroying (I.D.) titration (Stone, 1949a).

Enzymic activity of virus was tested by its ability to destroy the inhibitory power of ovomucin. Doubling dilutions of virus were prepared in 0·25 ml. volumes of calcium acetate saline, and to each was added an equal volume of a standard dilution of ovomucin containing 5 to 10 inhibitory doses (each dose being capable of inhibiting agglutination by 5 A.D. of the appropriate indicator virus). After incubation for one hour at 37° C., the mixtures were heated at 65° C. for 30 minutes to destroy the virus haemagglutinin, then cooled to room temperature. One drop of the indicator virus, containing 5 A.D., was added to each tube, followed after 30 minutes at room temperature by 0·25 ml. of a 1 per cent suspension of fowl cells. The cells were allowed to settle at room temperature, the end-point being taken as the one plus degree of partial agglutination. Presence of agglutination indicates destruction of inhibitor and hence enzymic activity of the virus.

Inoculation of eggs.

Eleven or twelve day embryos were inoculated allantoically with 0·05 ml. volumes of virus diluted in horse-serum saline, and the allantoic fluids harvested after incubation for 42 hours at 35° C.

Egg infectivity titration.

Serial tenfold dilutions of virus were prepared in 10 per cent horse-serum saline, and 0·05 ml. volumes inoculated allantoically into groups of five or six 11- or 12-day embryos. Following incubation for 42 hours at 35° C. the allantoic fluids were tested for haemagglutinin by adding one drop to 0·25 ml. of 1 per cent fowl cells. The 50 per cent infectivity end-point ($I.D._{50}$) is the dilution of virus which will infect 50 per cent of the inoculated eggs.

Neutralization of virus in ovo.

For this test ferret antisera were used which had been obtained by sterile cardiac puncture and stored without preservative at −10° C. Equal volumes of serum and virus, appropriately diluted in horse-serum saline, were mixed and allowed to stand for one hour at 4° C. Of the mixture 0·05 ml. volumes were then inoculated allantoically into groups of six 11- or 12-day embryos. The eggs were incubated, harvested and tested for agglutinins, as in egg infectivity titrations.

Intranasal inoculation of mice.

Groups of 9-weeks-old mice were lightly anaesthetized with a mixture of 2 parts ether and 1 part chloroform, and inoculated intranasally with 0·05 ml. of freshly harvested virus, diluted as required, in horse-serum saline.

Interference experiments.

The technique has been described previously (Isaacs and Edney, 1950*a*). Groups of six 11- or 12-day embryos were inoculated suballantoically with heat inactivated (interfering) virus. The eggs were incubated for one hour at 37° C. and then inoculated with 0·05 ml. of challenge virus appropriately diluted. Controls were inoculated with challenge virus only. The eggs were harvested and titrated individually for their haemagglutinin content. The tables in the text show the geometric mean haemagglutinin titres of the group of six eggs ± the standard errors.

EXPERIMENTAL.

The fowl cell receptor gradient.

Our first recognition of a variant came from experiments on the fowl cell receptor gradient.

Hirst (1942*a*) showed that following adsorption of virus to and elution from red cells, the cells were no longer able to adsorb or be agglutinated by fresh virus, i.e. the red cells receptors had been removed. Burnet, McCrea and Stone (1946) found that different virus strains varied in the extent to which they were able to remove red cell receptors, and they established the existence of a gradient of action. A virus " early " in the gradient, e.g. Newcastle disease virus (NDV),

removed red cell receptors to which homologous virus could attach, but still left receptors to which any virus " later " in the gradient could be adsorbed. However, a virus late in the gradient, e.g. swine influenza virus, removed receptors for homologous virus and for all viruses earlier in the gradient. At the same time it was shown that the gradient effect could be largely reproduced by the action of the *V. cholerae* enzyme, RDE. Red cells treated with large amounts of RDE were rendered inagglutinable by all strains of influenza virus. If smaller graded amounts of RDE were used it was found that the agglutinability of cells by different virus strains was removed in a definite order, which corresponded roughly to that of virus action.

Normal and variant MEL viruses were compared in their behaviour on agglutination of red cells. " Normal " refers to the characters usually associated with MEL virus, and " variant " to those of the variant. In the following experiment normal and variant MEL viruses were tested for their agglutination of RDE-treated red cells and the results read when the cells had settled at room temperature. A typical titration is shown in Table I.

TABLE I.—*Agglutination of RDE-treated Red Cells by Normal and Variant MEL Viruses.*

Virus.	RDE dilution.						
	100.	200.	400.	800.	1600.	3200.	Saline.
Normal MEL	−	−	++	++	++	++	++
Variant MEL	−	−	−	−	−	+	++

The signs indicate the degree of agglutination of the red cells ; 5 A.D. of each virus were used.

It can be seen that an amount of RDE which removes red cell receptors for the variant MEL still leaves the cells agglutinable by normal MEL virus, i.e. the variant MEL is " earlier " in the receptor gradient than the normal virus. The same experiment with human in place of fowl cells gave a similar result.

Action of variant and normal MEL viruses on red cells.

When human red cells are incubated at 37° C. with MEL virus until the cells are stabilized, i.e., they no longer agglutinate when suspended in saline, it is found that they are not agglutinated by a standard dose (5 A.D.) of a number of strains, including NDV, PR8 and homologous MEL virus (Burnet, McCrea and Stone, 1946). Thus, MEL has removed receptors for NDV, PR8 and homologous virus. When the cells were treated in similar fashion with variant MEL, the receptors for homologous virus were removed, but not for NDV, normal MEL and PR8.

Hanig (1948) found that following adsorption and elution of PR8, red cells showed a decreased electrophoretic mobility compared with normal red cells. Ada and Stone (1950*a*) have recently shown that red cells treated with different virus strains show a behaviour on electrophoresis which is similar in most respects to the receptor gradient pattern ; in general, the electrophoretic mobility is reduced least by viruses " early " and most by viruses late in the receptor gradient. It was expected, therefore, that variant MEL, which was " earlier " in the receptor gradient than normal MEL, would reduce the electrophoretic mobility of red cells less than normal MEL. This was tested experimentally by examining

the mobility of cells treated with variant MEL for 24 hours at 37° C., with the results shown in Table II.

TABLE II.—*Effect of Treating Human Red Cells with Variant and Normal MEL.*

Cells.	Agglutinability lost for.—	Electrophoretic mobility—u/sec./v./cm.		
		4 hours.	8 hours.	24 hours.
Normal	—	1·30	1·30	1·30
Treated with variant MEL	Variant MEL	1·22	1·21	1·12
Treated with normal MEL	Variant MEL, NDV, normal MEL and PR8	0·78	0·78	0·78

0·05 ml. of packed cells were treated with 4000 A.D. of each virus. Samples were taken after incubation at 37° C. for the times shown. The change in agglutinability was constant in all samples. Cells were stabilized with homologous antiserum. Cells treated with variant MEL were spontaneously stable after 4 hours at 37° C.

It can be seen that, in accordance with the results of Ada and Stone, there is a good correlation with these viruses between the extent to which the electrophoretic mobility of cells is reduced and the loss of agglutinability for different strains.

Action of variant and normal MEL on ovomucin.

Gottschalk and Lind (1949) have shown that ovomucin inhibits agglutination by heated LEE virus, and that the inhibitory power of ovomucin is markedly reduced by active MEL and LEE viruses. A comparison was made of the inhibitor-destroying (I.D.) power of variant and normal MEL viruses. The I.D. titration of Stone (1949*a*) was used. In this, a standard inhibitory dose of ovomucin (5 inhibitory doses) is incubated for one hour at 37° C. with falling dilutions of virus ; the highest dilution of virus which inactivates the inhibitor is taken as the I.D. titre. The results of a typical titration are shown in Table III.

TABLE III.—*Inhibitor-destroying (I.D.) Titrations of Variant and Normal MEL.*

Virus.	Haemagglutinin titre.	I.D. titre.
Variant MEL	480	<10
Normal MEL	960	480

Indicator virus : 6 A.D. of heated normal MEL.

Characteristically, the normal MEL gave a ratio of I.D. titre to haemagglutinin titre of 0·5 to 1 approximately. The ratio with variant MEL was always extremely low, indicating a low enzymic action.

A second method of showing this difference is by the technique of Burnet (1949). Here, 1000 inhibitory doses of ovomucin were incubated with 20 agglutinating doses of test virus for 18 hours at 37° C. The residual inhibitory titre was then estimated. It was found that the inhibitory titre of ovomucin against heated normal MEL was completely reduced by normal MEL, but only slightly by variant MEL. However, both viruses destroyed inhibitor for heated variant MEL and heated LEE.

Of the tests described two were selected which gave the most easily read

results. They were the red cell gradient position with RDE-treated cells (Table I) and the I.D. titration (Table III). When tests of the characters of an infected allantoic fluid were required, the two experiments were carried out in parallel along with appropriate controls. The results gave excellent agreement, and there was no difficulty in deciding whether a particular fluid behaved like variant or normal MEL.

Similarities between variant and normal MEL.

When the existence of a virus with the properties just described was first recognized, the possibility of contamination of our laboratory stocks was considered. However, it was soon clear that no other virus in the laboratory had the biological characteristics of this strain. It was also found that the variant had retained a number of properties of the original strain. Thus, the variant agglutinated fowl, human and mouse cells to normal titre, although the agglutination tended to slip readily at room temperature. The virus gave infectivity titres in eggs of the same order as normal MEL virus. The resistance to heat of the virus haemagglutinin was the same in normal and variant MEL ; heat resistance has recently been used as a differential diagnostic feature in this laboratory (Burnet and Lind, 1950).

One unusual characteristic of MEL virus is its behaviour on heating at 56° C. Stone (1949*a*) has shown that heating allantoic fluid preparations of MEL virus at 56° C. for 30 minutes does not transform the virus into an " indicator " for various mucoids, i.e. agglutination of red cells by the heated virus is not readily inhibited by ovomucin or other mucoids. However, if the virus is diluted in citrate-borate buffer, pH 8·5, and then heated at 56° C. for 30 minutes it is transformed into an indicator. It was found that variant MEL behaved in the same way as normal MEL on heating. The results of inhibitor titrations of ovomucin with variant and normal MEL viruses heated as described are shown in Table IV.

TABLE IV.—*Inhibitory Titres of Ovomucin with MEL Viruses Heated at* 56° *C.*

Treatment of virus.	Inhibitory titre of ovomucin for—	
	Variant MEL.	Normal MEL.
Unheated . . .	<100	<100
Heated at 56° C. for 30 min. . .	<100	<100
Heated at 56° C. for 30 min. in citrate-borate buffer . .	17,000	18,000

In each case 5 A.D. of virus were used.

The table shows that the variant and normal MEL behave alike on heating. The variant MEL was also tested for its serological behaviour in antihaemagglutinin titrations with ferret antiserum against stock MEL strain. Twelve fluids were found to give the same antihaemagglutinin titre as that given by normal MEL virus. However, since it seemed likely that the altered behaviour of the variant MEL towards red cells might be associated with changes in the surface structure of the virus, a more detailed investigation of its serology was carried out.

Serological reactions of variant MEL.

Ferret antisera were prepared against variant and normal MEL viruses ("Methods"). For this it was necessary to use a pure culture of normal MEL which did not contain any appreciable numbers of variant particles; the limiting dilution method used is described in the following paper (Isaacs and Edney, 1950*c*).

The antisera were tested in antihaemagglutinin titrations against normal and variant MEL. The results are shown in Table V; controls of normal ferret serum are included.

TABLE V.—*Antihaemagglutinin Titres of Antisera to Variant and Normal MEL Viruses.*

Serum.	Antihaemagglutinin titre.	
	Variant MEL virus.	Normal MEL virus.
V. variant MEL	3,200	150
V. normal MEL	700	1,200
Normal ferret serum	<100	<100

5 A.D. of each virus were used.

It can be seen that antiserum to normal MEL inhibited both viruses to almost the same titre. However, antiserum to variant MEL inhibited homologous virus to a much higher titre than it did the normal MEL.

The antisera were also tested for their ability to neutralize the viruses *in ovo* (Hirst, 1942*b*). In this test the viruses were diluted serially in 10-fold steps. The sera were diluted 1 in 20 and mixed with an equal volume of virus dilution. After one hour's incubation at 4° C., 0·05 ml. volumes were inoculated sub-allantoically into 11-day embryos as described in "Methods." The results are shown in Table VI, which gives the amounts of virus neutralized at the 50 per cent end-point by each serum.

TABLE VI.—*Neutralization of Variant and Normal MEL* in ovo *by MEL Antisera.*

Serum.	Amount of virus neutralized.	
	Variant MEL.	Normal MEL.
V. variant MEL	10^{6} I.D.$_{50}$	$<10^{1.3}$ I.D.$_{50}$
V. normal MEL	$10^{2.3}$ I.D.$_{50}$	$10^{6.3}$ I.D.$_{50}$

Sera diluted 1 : 20. Amount of virus neutralized at 50 per cent end-point is shown.

These results give a quantitatively different picture from those shown in Table V with the same viruses and antisera. However, *in ovo* neutralization tests are known to be more sensitive than antihaemagglutinin titrations in the detection of antigenic differences between strains. Burnet (1943) has shown that the type of neutralization obtained with influenza virus and antiserum in the allantoic cavity follows the equation $V = b\,S^{a}$, where V is the concentration of virus, S the concentration of serum, a and b are constants and a has a value of 3–4. Hence a thousand-fold difference in the amounts of virus which two

sera can neutralize implies a less than tenfold difference between the antibody content of the sera. The antigenic differences between normal and variant MEL do, however, allow the use of antiserum in separating mixtures of the two viruses.

Virulence of variant MEL for mice.

Fresh cultures of variant and normal MEL virus were prepared; they had approximately the same haemagglutinin titre. On the day of harvesting each virus was diluted 10^{-2} and inoculated intranasally into groups of ten 9-weeks-old mice.

The mice inoculated with normal MEL were all dead within six days; all lungs showed complete consolidation. The mice inoculated with variant MEL were killed on the seventh day. The lung-lesion scores are shown in Table VII.

TABLE VII.—*Lung Lesions of Mice Inoculated with Variant and Normal MEL.*

Virus.	Dilution of inoculum.	Per cent lungs consolidated.
Variant MEL	10^{-2}	10 ± 4 per cent
Normal MEL	10^{-2}	100 per cent

The amount of consolidation is expressed as the arithmetic mean $\pm$ standard error of the group. The degree of consolidation was estimated by inspection, and the figures given for the variant MEL group are rough approximations only.

In addition to these mice two further groups of ten mice each were also inoculated at this time with variant MEL 10^{-2}, and one group with normal MEL diluted 10^{-6}. These mice were intended for study of the serological and immunological response to inoculation with variant MEL, as its low pathogenicity, shown in Table VII, had been anticipated from the results of a preliminary experiment. Between the eighth and fourteenth day after inoculation, however, 10 out of 19 mice inoculated with variant MEL died with complete consolidation of the lungs. Four out of 10 mice inoculated with normal MEL 10^{-6} died in the same time with complete consolidation of lungs. The remaining mice were killed on the fourteenth day, and showed a similar distribution of lung lesions in the two groups. Both groups showed similar development of antibody to variant and normal MEL, the titres against variant MEL being slightly higher in each case.

It appears from these results that variant MEL diluted 10^{-2} had a lower pathogenicity in mice than normal MEL diluted 10^{-2}, but a similar action to normal MEL diluted 10^{-6}. The possibility that the results obtained with variant MEL might be due to mixture of a non-pathogenic variant with a small proportion of pathogenic normal MEL particles is discussed later.

The interference phenomenon with variant and normal MEL viruses.

We have been interested recently in the interference which results between inactive and active influenza viruses. A stock culture of MEL virus was used in most of our experiments (Isaacs and Edney, 1950*a*). It now appears that this culture contains roughly equal amounts of two components—normal MEL which shows the *in vitro* characters of the mixture, and variant MEL whose negative characters are not detected in the mixture. It was of interest to see whether

pure cultures of variant and normal MEL behaved similarly to the mixed stock.

The technique of interference experiments has been described previously. The interfering virus was prepared by heating infected allantoic fluid at 56° C. for one hour in citrate-borate buffer. This virus, which is non-infective, was then injected allantoically into groups of six 11-day eggs. After one hour's incubation the challenge virus was inoculated into these and into similar groups of control eggs. The eggs were incubated for 42 hours at 35° C., when the allantoic fluids were harvested individually and their haemagglutinins titrated. Two experiments of this kind are shown in Tables VIII and IX, where the figures give the geometric mean haemagglutinin titres ± standard error of the appropriate groups of eggs.

TABLE VIII.—*Interference with Inactive Normal MEL* versus *Active Variant and Normal MEL.*

Interfering virus.	Challenge virus. Variant MEL. 10^{-6}.	Variant MEL. 10^{-2}.	Normal MEL. 10^{-6}.	Normal MEL. 10^{-2}.
Heated normal MEL	13±6	8±3	140±51	224±13
Nil control	140±100	176±25	960	560±35

Interfering virus: 480 A.D. of heated normal MEL. The figures show the geometric mean haemagglutinin titre ± standard error of the group of eggs.

TABLE IX.—*Interference with Inactive Variant MEL* versus *Active Variant and Normal MEL.*

Interfering virus.	Challenge virus. Variant MEL. 10^{-5}.	Variant MEL. 10^{0}.	Normal MEL. 10^{-5}.	Normal MEL. 10^{0}.
Heated variant MEL	20±15	134±13	304±65	304±42
Nil control	380±52	560±80	840±103	560±90

Interfering virus: 280 A.D. of heated variant MEL. The figures show the geometric mean haemagglutinin titre ± standard error of the group of eggs.

The results in Table VIII show that the same inoculum of heated normal MEL interferes more readily with variant than with normal MEL. The difference is more marked with the larger inoculum of challenge virus. An experiment with heated variant MEL as interfering agent (Table IX) gave a similar result, i.e. 280 A.D. of heated variant MEL interfered more readily with variant than with normal MEL This fact was confirmed in a third experiment, where each virus was used in the inactive form as interfering agent, and in the active form in two different dilutions, as challenge virus. The results suggest that variant and no mal MEL differ in their reactions as challenge viruses in an interference experiment, but do not differ as interfering agents.

Variants of other strains of influenza virus.

When the presence of two genetically distinct forms of MEL virus was first recognized a search was made for similar variants in other stock cultures of influenza viruses. The strains examined were PR8, LEE and Ocean Island viruses.

PR8.—By passage of stock PR8 at limiting dilution two types of PR8 virus were found which differed in their positions in the fowl cell receptor gradient. The difference was not so marked as that shown in Table I for MEL virus, the PR8 types being separated by only one RDE dilution. However, the difference was maintained in four successive passages of eah type at a 10^{-6} dilution.

The two types were then compared in the tests which were carried out with MEL virus variant. No differences were detected between them, however, in the following experiments—inhibitor destroying titrations, removal of human red cell receptors by virus action, effect on the electrophoretic mobility of red cells and interference experiments The difference in gradient position found in the two PR8 types was thus found to be independent of detectable differences in the other reactions listed.

LEE.—Eighty-one eggs were injected with stock LEE virus at limiting dilution, as described in the following paper. Thirty-nine showed virus multiplication, and were tested for their position in the fowl cell receptor gradient. All the fluids behaved alike in this test, as did 11 of these fluids on which inhibitor-destroying titrations were carried out.

Ocean Island.—One hundred and eighty-eight eggs were injected with stock Ocean Island virus at limiting dilution. Forty-nine showed virus multiplication, and all gave the same reactions in the fowl cell receptor gradient tests.

Two of the four influenza virus stocks examined have therefore been found heterogeneous. However, the tests used do not exclude heterogeneity in other characters, and in addition, it is only practicable to detect a mixture by this method when the ratio of one virus to the other is close to 1 : 1.

DISCUSSION.

The study of virus variants seems worth while as a means of comparing alterations in biological properties with changes in pathogenicity ; as information about further strains becomes available, a correlation of these factors may be attempted. A group of changes observed with MEL virus variant is concerned with the reactions of the virus towards red cells and inhibitors of haemagglutination. In a general discussion on virus haemagglutination and its inhibition by mucins and mucoids, Anderson, Burnet, Fazekas de St. Groth, McCrea and Stone (1948) put forward the following hypothesis : The red cell surface contains virus receptors made up predominantly of mucopolysaccharide molecules. Following adsorption of virus to the cells, progressive enzymic action reduces the amount of receptor substance, and concomitantly, the adsorptive affinity for viruses of the influenza group diminishes. At a certain stage of enzymic action, insufficient receptor substance is present to maintain the forces required for adsorption and the virus elutes from the cell. A virus " early " in the gradient is adsorbed to receptors, from which it elutes at a stage when receptors for viruses " later " in the gradient are still left. In the present experiments it was found that after minimal action of RDE on red cells, receptors for variant MEL were removed while those for normal MEL remained ; and that following enzymic action of variant MEL on normal red cells there was less removal of receptors than with normal MEL. Thus variant MEL is earlier in the receptor gradient than normal MEL. There was also less alteration of the electrophoretic mobility of red cells by variant than by normal MEL, a result which was to be expected on the basis of Ada and Stone's (1950*a*) findings. Similarly, the lower enzymic action of

variant MEL on ovomucin and the greater sensitivity of heated variant MEL as an indicator strain show that the variant strain is " earlier " than the normal virus in the ovomucin inhibitor gradient (Stone, 1949*b*).

Alteration in the adsorptive behaviour of variant MEL towards red cells and ovomucin may have occurred as a result of a mutation involving changes in the surface pattern of the virus. Study of the antigenic characters of the normal and variant MEL lends support to this idea. Antiserum prepared against normal MEL neutralized variant MEL to a lesser degree than homologous virus, but the difference is not great. However, antiserum to variant MEL had little neutralizing action towards normal MEL. If this implies a mutation by loss it is of interest that the loss in antigenic material was associated with reduction in mouse pathogenicity and ability to overcome interference.

The difference in behaviour of variant and normal MEL in interference experiments invites the question of the mechanism involved. It has been shown (Isaacs and Edney, 1950*b*) that under the conditions of the present experiments interference is an intracellular process. It seems that variant MEL does not differ from the normal form in its ability to multiply in the allantoic cavity or in its power to interfere with the multiplication of other viruses, but only in its reduced capacity for " overcoming " intracellular interference by inactive virus. The mechanism is not understood, but it is clear that the use of pure cultures is desirable in studies on interference.

The low pathogenicity of variant MEL for mice was of particular interest. The actual lesions and late deaths observed in the mice inoculated with variant MEL may have been due to the presence of small numbers of normal MEL particles in the inoculum, which might have developed by back mutation of variant to the normal form. In the following paper it is shown that back mutation can be demonstrated under suitable conditions. If this explanation is true, the variant *per se* would have a very low mouse pathogenicity. This point requires further investigation in connection with studies on adaptation of influenza viruses to mice.

SUMMARY.

1. A variant of Melbourne influenza virus (MEL) was isolated from a stock culture by inoculating eggs at limiting dilution.

2. The variant was earlier in the red cells receptor gradient than the normal virus and had a reduced enzymic activity on ovomucin, compared with the normal virus.

3. Ferret antisera showed serological differences between the variant and normal MEL.

4. The variant had a lower pathogenicity in mice than the normal virus.

5. Variant MEL was more sensitive to interference than normal MEL, although the viruses were equally effective as interfering agents.

6. Three other influenza virus stocks were examined for inhomogeneity by the limiting dilution method. Two types of PR8 virus, which differed in red cell gradient position, were isolated in this way.

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VARIATION IN LABORATORY STOCKS OF INFLUENZA VIRUSES: GENETIC ASPECTS OF THE VARIATIONS.

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THE methods of cultivation of animal viruses in the laboratory are at present similar to those used for bacteria before solid media were discovered. In each case passage involves the transfer of a number of organisms, which may differ in their individual characteristics, from one medium to a fresh one. Clearly the accurate study of viruses requires some means of separating a mixture into its components and of growing these in pure culture.

During studies on the growth of Melbourne strain of influenza virus A in fertile eggs, we passaged our stock strain at a dilution of 10^{-7} in order to prepare fresh seed which would not give rise to auto-interference, a procedure which had been suggested by Henle and Henle (1949). This virus and allantoic fluids descendent from it gave the characters of the variant MEL just described. Further investigation showed that when our stock Melbourne virus was injected into eggs in dilutions from 10^{0} to 10^{-6} the resulting fluids behaved like normal MEL. A 10^{-7} dilution of our stock virus was the limiting dilution. When a batch of eggs was inoculated with this dilution some showed no growth of virus, some behaved like normal MEL, and some like variant MEL. It seemed, therefore, that our stock culture contained roughly equal numbers of normal and variant MEL particles. Passage at low dilution would give a seed containing both types, and under these conditions the characters of the normal virus showed. At limiting dilution, however, infection would be initiated in some eggs by a single infective virus unit, allowing separation of the stock into pure cultures of each of its constituents. The standard procedure for preparation of virus in this laboratory is inoculation of eggs with stock virus at a dilution of 10^{-4}; this would account for the fact that only the normal MEL characters had been recognized hitherto.

Passage at limiting dilution does therefore allow the production of pure clones of viruses, which may be of use in the study of a number of virus problems, and which have been essential for the examination of the mutations described in this paper. A similar conclusion was reached independently by Burnet (1950). Since the methods used in isolating the mutants required a knowledge of the properties of the viruses, these were described first in the preceding paper (Isaacs and Edney, 1950).

MATERIALS AND METHODS.

Most materials and methods are described in the preceding paper.

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Storage of stock virus cultures.

Stock virus cultures for general laboratory use are kept at −10° C. in the form of infected allantoic or amniotic fluid plus 33 per cent glycerol. When seed virus is required, 0·05 ml. is withdrawn and made up to a 10^{-4} dilution in horse-serum saline. Of this, 0·05 ml. volumes are then inoculated allantoically into groups of 11-day embryos.

RDE treatment of eggs.

Eleven-day embryos were inoculated allantoically with 0·5 ml. RDE and incubated for 2 hours at 37° C. to allow the enzyme to act on cell receptors. The test virus was then injected allantoically and the eggs incubated for 42 hours at 35° C., when the allantoic fluids were harvested. All titrations involving red cells were carried out with 2 per cent citrate-saline as diluent, in place of normal saline, in order to reduce further action of RDE on the test cells.

EXPERIMENTAL.

Limiting dilution.

Previously, this term has been used to describe the highest dilution in an infectivity titration at which virus growth occurs (e.g. Burnet and Bull, 1943). In the present work it is used in a more specialized sense. Limiting dilution is defined as the end-point of an infectivity titration at which less than 50 per cent of the inoculated eggs show growth of virus. The assumption is that when less than 50 per cent of the inoculated eggs show growth of virus there is a certain probability that growth in some of the eggs will be initiated by a single infective virus unit, and hence that " pure " cultures of virus will be produced. This probability can be increased as much as required by choosing a limiting dilution end-point as high as necessary, e.g. the end-point dilution at which 5 per cent of the inoculated eggs show growth of virus. In the majority of our experiments an end-point between 25 and 50 per cent was chosen.

Passage of stock MEL at limiting dilution.

When the laboratory stock MEL was injected into 12 eggs at a dilution of 10^{-7}, five showed no growth of virus, five showed growth of normal type virus, and two growth of variant virus. The results are represented diagrammatically in Fig. 1. For simplicity, an allantoic fluid showing the characters of normal MEL is described as " *M* " ; a fluid showing the characters of variant MEL as " *m.*" The tests used to decide whether a fluid was " *M* " or " *m* " were the receptor gradient of RDE-treated cells and I.D. titrations on ovomucin.

The points which can be seen in Fig. 1 are that separation of the stock into two constituents was possible at limiting dilution. Once *m* virus was isolated it bred true at whatever dilution it was passed. Similarly, *M* virus obtained at limiting dilution and inoculated into a group of 30 eggs at limiting dilution gave 17 positive fluids, all of which were *M*. Limiting dilution, like plating out bacteria, can therefore be used to isolate individual clones and to test their purity.

Effect of inoculation into eggs of a mixture of M *and* m.

If this interpretation of the results of growing stock MEL was correct, inoculation of eggs with a mixture of equal parts of *M* and *m* should give fluids showing

the *M* character. This was found to be so experimentally. When 10^7 I.D.$_{50}$ of each virus were mixed and inoculated into groups of eggs, the fluids harvested after 42 hours' incubation all showed the *M* character. A similar experiment with 10^3 I.D.$_{50}$ of each virus gave the same result. The effect is the same as that produced by inoculation of stock MEL at low dilution.

In a second group of experiments *M* and *m* were mixed in the proportion $M : m :: 1 : 10^4$ and inoculated into groups of eggs. In this case the fluids showed characters intermediate between *M* and *m*, i.e. low but measurable enzymic activity in the I.D. titration and intermediate fowl cell gradient position. From this it can be inferred that in order to produce cultures showing phenotypic *m* characters it is necessary to have a proportion of *M* virus of less than 10^{-4} in the seed.

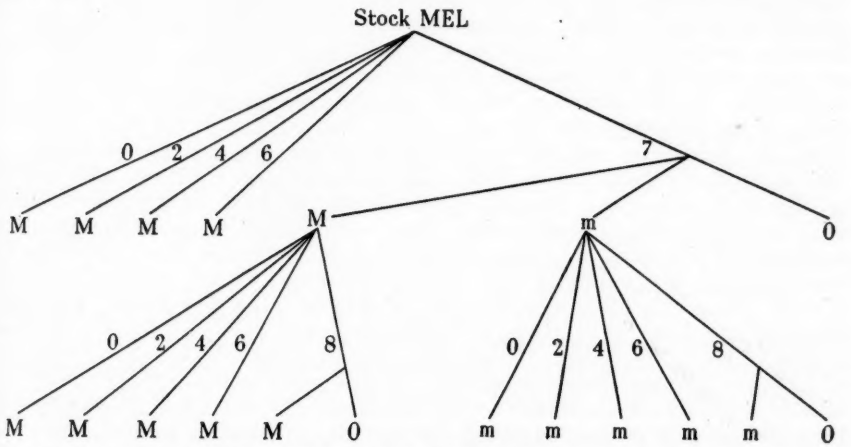

FIG. 1.—Passage of stock MEL virus at limiting dilution. Figures indicate the $\log_{10}$ dilution at which the passage was made.

The characters by which *M* are recognized are positive, while the *m* characters are negative. A mixture of equal parts (by haemagglutinin titre) of *M* and *m* *in vitro* behaved like *M* in its gradient position ; also the enzymic activity of the mixture is governed by its content of the actively enzymic *M*.

Attempts to isolate m *from an artificial mixture of* M *and* m.

When pure clones of *M* were available, it was of interest to see whether *m* mutants were being produced by *M* during its growth in the allantoic cavity. Inoculation of a very large group of eggs at limiting dilution was impracticable, so that some means was required of growing the viruses under conditions which would favour the multiplication of *m*. Preliminary experiments were carried out on mixtures of *M* and *m* prepared *in vitro* in known amounts.

Absorption with RDE-treated red cells.—RDE-treated red cells offered a possible means of separating *M* and *m*, since there was a zone of RDE-treated cell concentrations at which *M* was adsorbed to cells, but not *m*. Adsorption had to be carried out in the cold, however, in order to reduce enzymic elution of

virus from the cells. The receptor gradient tests with *M* and *m* had previously been carried out at room temperature; the experiment was therefore repeated with the reagents chilled and the cells allowed to settle at 0° C. The difference between *M* and *m* was found to be less at 0° C. (one RDE dilution) than at room temperature, suggesting that part of the difference at room temperature could be accounted for by rapid elution of *m* from the RDE-treated cells. At 0° C. it was found that cells treated with 1/200 RDE were agglutinated by *M*, but not by *m*.

Accordingly, a mixture of 10^8 I.D.$_{50}$ of *M* and 10^4 I.D.$_{50}$ of *m* was prepared in 1 ml. of horse serum saline, and to this was added 0·1 ml. of packed fowl red cells which had been treated with 1/200 RDE. After standing for 20 minutes at 0° C. the mixture was centrifuged in the cold and the supernatant removed. Two further adsorptions with the same amount of RDE-treated red cells were then carried out at 0° C. Finally 0·05 ml. volumes of the supernatant were inoculated into eggs undiluted and 10^{-3} Only *M* virus grew in these eggs. The experiment was then repeated, this time with cells treated with 1/400 RDE; the same result was found. In the second experiment a control was included of the egg infectivity of the mixture before and after adsorption. It was found that the infectivity was reduced by only one $\log_{10}$ unit, i.e. 90 per cent, by three absorptions. This method was therefore not efficient enough to separate a mixture of *M* and *m*.

Use of M *antiserum.*—Owing to the difference between *in ovo* neutralization of *M* and *m*, it seemed possible that *M* antiserum might be useful in separating a mixture of *M* and *m*. In this case a less exacting test was carried out. Mixtures were prepared of equal parts of *M* and *m* containing 10^3, 10^4, 10^5 and 10^6 I.D.$_{50}$ of each virus. To each tube 0·05 ml. of *M* antiserum was added, and after standing one hour at 0° C. 0·05 ml. volumes were injected allantoically into groups of six 11-day eggs. In the first three groups 8 fluids showed growth of virus and all were phenotypically *m*. In the group inoculated with 10^6 I.D.$_{50}$ of each virus, 1 fluid was *m* and 4 were intermediate in character, suggesting that both viruses were present, although the majority of particles were *m*. In view of these results, *M* antiserum was used to investigate the possibility of *M*—→*m* mutation.

Growth of M *in increasing concentrations of homologous antiserum.*

The method adopted for attempting to isolate *m* mutants was as follows: pure *M* virus was inoculated into a group of eggs in the presence of that concentration of *M* antiserum which produced approximately 75 per cent neutralization. Where virus showed growth at this end-point, the fluids were harvested and again inoculated into further eggs in the presence of an increased relative concentration of antiserum. In this way it was possible to select at each passage those virus particles which were less efficiently neutralized by *M* antiserum *in ovo*.

A " pure " culture of *M* virus was used, i.e. one which did not contain appreciable amounts of *m*. It had been isolated from stock MEL at limiting dilution, passaged once at 10^{-9}, tested for purity at limiting dilution, and finally passaged at 10^{-5}. For the first serum passage in this experiment the virus was diluted 10^{-2}, and added to an equal volume of *M* antiserum serially diluted 1 : 10 to 1 : 320. After one hour at 0° C., 0·05 ml. volumes of each mixture were inoculated allantoically into groups of eggs. Altogether 3 out of 11 fluids from the 1 : 20 antiserum and 1 out of 6 from the 1 : 10 antiserum groups showed

growth of virus. These four fluids were then sub-inoculated in a 10^{-2} dilution along with *M* antiserum diluted 1 : 10 and 1 : 20 as before. Two of these fluids behaved in the same way as at the first pass, i.e. growth occurred in only 1 out of 6 eggs inoculated; they may have represented chance growth of *M* virus near the end point. The other two fluids showed growth in all eggs in both the 1 : 10 and 1 : 20 antiserum groups. This implies development of an increased resistance to *M* antiserum *in ovo*, and the fluids were therefore passaged further. With two more serum passages both these viruses diluted 10^{-6} when mixed with *M* antiserum 1 : 20 showed growth in 6 out of 6 eggs. This virus has been labelled *Ms* to indicate its increased resistance to neutralization *in ovo* by *M* antiserum.

At each passage the viruses were tested for their position in the fowl cell receptor gradient; all viruses showed the *M* character throughout. It appears, therefore, that as a result of passage of *M* with homologous antiserum a mutant, *Ms*, was isolated which was at least ten thousand times more resistant to neutralization by *M* antiserum *in ovo* than *M* virus, but which did not differ from *M* in its position in the fowl cell receptor gradient.

It was necessary to show that *Ms* was a mutant, and that it did not grow by adaptation only. *Ms*, obtained from the fourth serum passage, was therefore grown in the allantoic cavity in the absence of serum and tested by neutralization tests *in ovo* with *M* antiserum, in order to see whether the virus would retain its *Ms* character. After a preliminary pass at 10^{-6}, the virus was passaged serially both at 10^{-1} and 10^{-8} for four passages. At each pass the virus was tested by neutralization *in ovo* with *M* antiserum. In all cases the viruses diluted 10^{-4} to 10^{-6} were not neutralized by *M* antiserum 1/20, *in ovo*.

The *Ms* virus thus retained its character after five allantoic passages made in the absence of serum—a change characteristic of a mutation rather than an adaptation. Further, the *Ms* showed no tendency to revert to *M* when passaged at low dilution (10^{-1}).

Attempts to show mutation of m *to* M.

Stone (1948) has shown that injection of RDE prevents infection by influenza viruses in the allantoic cavity. The RDE was thought to act by removing the cell receptors required for adsorption of the virus. It seemed possible that treatment of the allantoic cavity with a critical concentration of RDE might remove receptors for *m*, while leaving *M* free to infect the cells.

This was tested in a preliminary experiment by inoculating a group of eggs with 0·5 ml. RDE. After 2 hours at 37° C. the eggs were injected with 10 $I.D._{50}$ of *M* + 10^6 $I.D._{50}$ of *m*, and incubated for 42 hours at 35° C. The fluids were harvested and their characters tested, dilutions being made in 2 per cent citrate in order to stop further RDE action. All fluids showed growth of *M*, while controls of the same inoculum without RDE gave *m* virus. RDE-treated eggs thus gave an opportunity to see whether mutation of *m* to *M* was occurring.

A pure culture of *m* virus was inoculated into RDE-treated eggs in amounts from $10^{3.3}$ to $10^{6.3}$ $I.D._{50}$. Control normal eggs were inoculated with the same amounts of virus. Infection by up to 10^4 $I.D._{50}$ of *m* was prevented by RDE. With greater amounts of virus in the seed, growth in RDE-treated eggs led to the production of *M* virus. The experiment was then repeated with fresh *m* virus obtained by passaging the above seed at limiting dilution; when this was inocu-

lated into RDE-treated eggs, *M* virus again grew. It was also found that in some eggs *m* and intermediate type virus was obtained, so that it is clear that the RDE-treatment did not prevent the growth of *m*, but only reduced it considerably. It is thus not possible to calculate the mutation rate of *m* to *M* by this means.

The *M* virus obtained from inoculation of RDE-treated eggs with *m* gave the same reactions as *M* in the receptor gradient and I.D. titrations on ovomucin, but differed from *M* serologically. It has therefore been labelled *Mr*.

Antihaemagglutinin titrations with MEL mutants.

The various mutant forms of MEL virus were tested for their behaviour in anti-haemagglutinin titrations with ferret antisera to *M* and *m* viruses.

Mr virus, derived from *m*, was found to contain about 50 per cent of *m* when first isolated. It was purified by passage at limiting dilution, and tested for purity

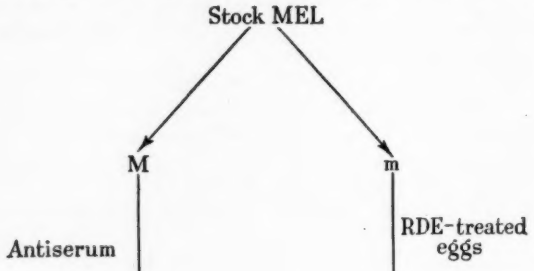

FIG. 2.—Diagram of types of MEL variants.

by a further limiting dilution pass which now showed no growth of *m* ; allantoic fluid from this pass was used to test the serology of *Mr*. *Ms* virus was also tested in the same way and no *m* was found present in the culture. In the final anti-haemagglutinin titrations, therefore, only pure clone viruses were used. Representative results of a number of titrations are shown in Table I ; each virus showed a constant pattern of behaviour in a series of tests.

TABLE I.—*Antihaemagglutinin Tests of Mutants of MEL.*

Virus.	Serum. Anti *M*.	Anti *m*.
M . . .	1600 .	150
m . . .	800 .	3200
Ms . . .	400 .	<100
Mr . . .	600 .	400

In each case 5 A.D. of virus were used.

Table I shows that *Ms* differs serologically from *M*, although as with *m*, the difference appears less marked than that shown by *in ovo* neutralization tests. *Mr* differs from *m*, although in this case part of the difference may be accounted for by the nature of the test. Since the change *m*—→*M* involves an increased

affinity of virus for the red cell, this might be reflected in a relative decrease in the affinity of virus for antibody. However, *Mr* and *m* show a reversal in their titres with antisera to *M* and *m*, and this is likely to be the result of a true serological change. The serological behaviour of *Mr* was also tested in a neutralization test *in ovo* with antisera to *M* and *m*. As might be expected from its serological behaviour *in vitro*, *Mr* diluted 10^{-4} to 10^{-6} was not neutralized by a 1/20 dilution of either *M* or *m* antiserum.

Ms and *Mr* therefore appear to be intermediate between *M* and *m* serologically, *Ms* being closer to its parent *M* and *Mr* to its parent m.

Relationship between MEL mutants.

The relationship between the various mutant forms is shown in Fig. 2.

There is at present no convenient experimental method of showing mutation of *Ms* to *Mr*, or of any other steps in the mutations. It is interesting to note that the results resemble in some ways those shown for mutants of T2, T4 and T6 bacteriophages by Hershey (1946).

DISCUSSION.

It appeared at first that two types of MEL virus existed, *M* and *m*, but further examination has shown two intermediate types, *Ms* and *Mr*. It is possible that further types may be found on closer analysis. Workers on bacteriophages have suggested that these organisms have a finite number of what would be equivalent to genes in higher organisms at a number of loci, each "gene" being capable of existence in two allelic forms (e.g. Hershey and Rotman, 1948). If this hypothesis is applied to influenza viruses, one or two or a number of "genes" might govern red cell gradient position, and the same would hold for other biological characters of the virus. Whether mutations in animal viruses occur in this way remains to be seen.

It is not yet known how the two types of MEL virus came to be present in the stock culture. It was assumed that *M* represented the parent strain, as its characters, particularly its mouse pathogenicity, are those generally associated with MEL virus. Also the earliest egg passage MEL virus available contained a higher proportion of *M* to *m* than a more recent culture. If this is so, it is difficult to account for the presence of *m* in such high numbers, unless it has a higher ability to survive than *M* under the conditions of storage of our stock cultures. A similar explanation was suggested by Findlay (1939) to account for the survival of a variant of the fibroma virus described by Andrewes (1936) and Shope (1936). In addition, Panthier, Cateigne and Hannoun (1948) have noted that cultures of PR8 virus stored at $-20°$ C. for 4 months showed a decreased egg infectivity titre with an increased mouse pathogenicity—a result which they attribute to selective survival of more virulent variants at the temperature of storage.

The mutation *M*—→*Ms* was of interest in view of unpublished experiments which one of us had carried out in 1948 along with Professor C. H. Stuart-Harris. At that time, strain PR8 was passaged in a similar way in the presence of increasing concentrations of homologous antiserum, and a mutant form was found which was more resistant to neutralization by antiserum *in ovo* than the original virus. If the phenomenon is a general one this may be a useful method of obtaining mutants for study.

The present experiments have dealt in a limited manner with only a small number of properties of the mutants. Further work on mutants may provide information on the characters associated with pathogenicity of influenza viruses.

The nature of the methods used in cultivating animal viruses has not favoured the laboratory study of mutants. This is partly the result of lack of pure virus cultures for initial study, and partly owing to the difficulty of isolating mutants from the parent stock. Passage at limiting dilution provides a method for obtaining pure virus clones and for testing the purity of the resulting cultures. A single passage at limiting dilution will not, of course, invariably give rise to pure clones, but the method can be adjusted to increase, by almost any factor required, the probability of a particular culture arising from a single infective unit, e.g. by choosing an end-point dilution at which less than 5 per cent of the inoculated eggs show growth of virus and repeating limiting dilution passages as often as required. Applied to a number of general problems of research on animal viruses, the technique may give results of considerable interest. The heterogeneity of laboratory stocks has not been confined to the strains described here, but has also been found by Burnet (1950, personal communication) for one Newcastle disease virus strain. In addition, the presence of mutants in laboratory stocks has been tested for mainly on one criterion—red cell gradient position. There is no reason to suppose that this is the locus at which mutations occur most commonly, or that the resulting heterogeneous virus stocks will be confined to this Institute.

SUMMARY.

1. Limiting dilution is defined and its use described in the separation of a stock Melbourne influenza virus culture into pure clones of its two constituents—normal MEL (*M*) and variant MEL (*m*).

2. Attempts to show mutation of *M* to *m* were not successful. Growth of *M* in increasing concentrations of homologous antiserum led to the production of a serum-resistant mutant, *Ms*.

3. Inoculation of a pure culture of *m* virus into RDE-treated eggs led to growth of a mutant, *Mr*, which resembled *M* in red cell gradient position and enzymic activity, but differed from it slightly in serological tests.

4. Limiting dilution, like plating out bacteria, provides a method for obtaining pure virus clones and for testing their purity.

We are deeply indebted to Professor F. M. Burnet for his advice and criticism. We should also like to thank Mr. G. L. Ada for carrying out the micro-electrophoresis measurements.

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THE APPLICATION OF THE CONGLUTINATING COMPLEMENT ABSORPTION TEST TO VIRUS SYSTEMS.

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HOLE AND COOMBS (1947*a*, 1947*b*, 1947*c*) have recently investigated the conglutinating complement absorption test (C.C.A.T.) which was originally developed by Streng (1910) from the principle of conglutination described by Bordet and Gay in 1906.

The experiments of Coombs and Hole (1948) and Blomfield, Coombs and Hole (1949) have shown that the animal species of complement used in a complement fixation test has an important bearing on the sensitivity of the reaction. The relative merits of only haemolytic complements had been investigated by earlier workers (Noguchi and Bronfenbrenner, 1911), but the development of the C.C.A.T. allows a comparison of horse, cat, pig, dog and even human conglutinating complements with guinea-pig haemolytic complement. Antisera to mallein and *S. pullorum* were investigated with these complements. Although the complement of choice varied somewhat with the species nature of antisera examined, the demonstrable antibody titres with horse conglutinating complement were in most instances higher than with any other complement and much higher than with guinea-pig complement. This was particularly the case with human antisera in which antibodies might be indetectable by the haemolytic complement fixation test (H.C.F.T.) with guinea-pig complement but were revealed to a high titre by the C.C.A.T. with horse complement.

The results appeared to be of practical significance and it was decided to investigate the use of the C.C.A.T. with horse complement in the diagnosis of other diseases.

The H.C.F.T. is an important serological procedure in the investigation of virus infections because difficulties in purification may make other techniques, such as agglutination and precipitation, impracticable. Using guinea-pig complement, antibodies have been detected in many viral and rickettsial diseases especially since cultivation of these agents in the developing egg has made potent antigens available.

It was therefore decided to perform experiments to compare the C.C.A.T. and H.C.F.T. in certain virus diseases, and for preliminary investigations psittacosis-lymphogranuloma venereum, vaccinia and influenza were chosen.

In 1943 Solovieff investigated the use of C.C.A.T. (using horse complement)

for the diagnosis of foot and mouth disease. He examined 1329 sera from different species of animals, ox, pig, guinea-pig, horse, rabbit and sheep. Some were from convalescent and some from hyperimmunized animals; normal sera were included as controls. Three hundred and ninety of these sera were also examined by the H.C.F.T. No description of the antigen is given and the sera were examined in one dilution only, but the results with the C.C.A.T. seemed very satisfactory and gave fewer doubtful reactions. Solovieff concluded that the C.C.A.T. was a reliable method for demonstrating antibodies in this disease.

Since our investigations commenced Wolfe and Kornfeld (1948) have published an account of the use of the C.C.A.T. in the diagnosis of Q fever in cattle. Although the fixation periods in the C.C.A.T. and H.C.F.T. were not comparable, their results suggested that the C.C.A.T. was both more specific and more sensitive.

This paper is a preliminary study on the application of the C.C.A.T. to virus diseases. It describes the difficulties which are encountered when horse complement is used with virus antigens and records some direct comparisons of the C.C.A.T. and H.C.F.T.

MATERIALS AND METHODS.

1. *Antigens.*

Psittacosis-lymphogranuloma venereum group.—This antigen was prepared from the yolk sacs of embryonated hens' eggs infected with psittacosis virus. The antigen was partially purified by sedimentation and differential centrifugation in an angle centrifuge as previously described (Bedson Barwell, King and Bishop, 1949). Since the final suspension was heated at 100° C. for 20 minutes it contained only the heat-stable antigen common to this group of viruses. As it has been used principally with lymphogranuloma-venereum antisera it is referred to as the L.G.V. antigen in this paper.

Vaccinia.—The vaccinia antigens were prepared from the chorioallantoic membranes of fertile hens' eggs. Eggs previously incubated for 11 days were inoculated with a dilution of vaccinia virus so as to give confluent lesions with as few deaths as possible when harvested after incubation for a further three days. The infected membranes were pooled, weighed, ground with saline in a Griffith grinder and allowed to extract at + 2° C. overnight. The suspension was then centrifuged at 2000 r.p.m. for 10 minutes and the supernatant fluid which was the crude antigen, was stored at − 20° C. without added preservative.

Influenza.—Dried mouse lungs infected with Influenza A (PR8) as issued by the World Influenza Centre at Hampstead were kindly sent to us by Dr. C. M.Chu.

2. *Antisera.*

Psittacosis L.G.V. Group.—These were from patients suspected of suffering from infection with L.G.V. virus or a virus of the psittacosis group.

Vaccinia.—Rabbit antivaccinia sera were obtained from animals which had been infected by scarification with a rabbit-adapted (Salaman) strain of vaccinia, and subsequently hyperimmunized by repeated intravenous injections of a purified elementary body suspension or subcutaneous injections of rabbit vaccinia crust suspensions.

Human antivaccinia sera were obtained after successful vaccination from a group of adults, most of whom had been vaccinated previously in infancy.

Influenza.—A pool of convalescent human Influenza A antisera and specimens from individual patients were kindly sent by Dr. A. P. Goffe, of the Virus Reference Laboratory at Colindale.

3. *Complements and other reagents.*

Guinea-pig complement was used in the H.C.F.T. while the complement in fresh horse serum was used for most of the conglutination tests. Cat and pig complements were also used in certain experiments, but previous investigations with mallein and *Salmonella* antigens had shown that these were less suitable than horse complement, particularly with human antisera. All complements were stored in approximately 1 ml. amounts at — 20° C. without chemical preservative.

The haemolytic and conglutinating indicator systems were those described by Hole and Coombs (1947*b*).

4. *Technique of tests.*

The techniques for performing the H.C.F.T. and C.C.A.T. were those described by Hole and Coombs (1947*b*) with the following modifications which were introduced for the purpose of working with live or dead viral antigens.

The unit volume of antigen, complement, antiserum, cells and either haemolysin or inactivated bovine serum was 0·1 ml. instead of 0·4 ml., giving a total volume of 0·5 ml. in each tube. This change was introduced in order to save antigen.

The period and temperature of incubation of the antigen with complement and antiserum varied and were the subject of experiments described later. The preliminary complement titration in all these tests was performed in the presence of the antigen under conditions identical with those used in the actual test.

The results of the conglutination tests were read after centrifugation at 2000 r.p.m. for one minute in a specially adapted centrifuge and resuspension of the deposited cells. Using the 0·5 ml. amount this resuspension is most effectively carried out by replacing the tubes in their racks and flicking the bottoms of the tubes 10 times as they hang by their rimmed necks. Unconglutinated cells form a homogeneous suspension, while conglutinated cells remain in large discrete clumps. The haemolytic tests were also centrifuged to allow a more accurate recording of degrees of haemolysis.

EXPERIMENTAL STUDY.

1. *Anticomplementary Activity of Virus Antigens for Horse Complement.*

A major difficulty which is encountered when using the H.C.F.T. in virus infections is the anticomplementary action of the crude tissue extracts which are the source of some virus antigens. This constituted an even greater problem in the C.C.A.T. because it was soon apparent that these extracts fixed horse complement non-specifically even more than they did guinea-pig complement.

In practice, this anticomplementary action can usually be overcome by sufficient dilution of the antigen. Such a diluted antigen may still give more sensitive specific fixation of horse complement than concentrated antigen used with guinea-pig complement. Nevertheless, for a direct comparison of the H.C.F.T. and the C.C.A.T. it was preferable to use antigens in the same range

of dilutions in both tests and to determine the optimal antigen concentration for each without the limitation caused by anticomplementary action. In the case of weak virus antigens this optimum is often the strongest concentration possible which is in turn limited mainly by the anticomplementary action of the antigens.

In these experiments we have examined various methods of treatment in order to remove the anticomplementary effect of crude antigens for horse complement.

(a) *L.G.V. Antigen.*

The L.G.V. antigen has been found to give the best results in the H.C.F.T. when it is used at a dilution of 1:4. Although at this strength it was not anti-

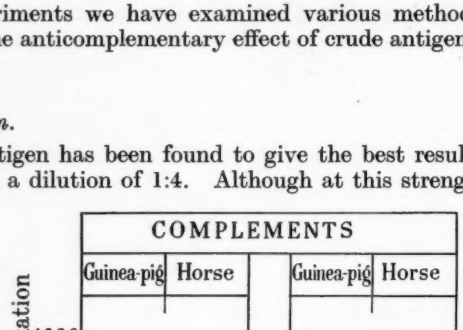

FIG. 1.—Titration of two human L.G.V. sera by the H.C.F.T. and C.C.A.T. using the antigen at dilutions of 1:4 and 1:80 respectively; the 1:80 dilution being necessitated by the anti-complementary effect of the antigen for horse complement.

Key to this and subsequent figures:

- Complete fixation of complement.
- Anticomplementary action of serum.
- Partial fixation of complement.
- No apparent fixation of complement. May indicate a prozone.

complementary for guinea-pig complement it was far too anticomplementary for horse complement to allow its use in the C.C.A.T. The antigen had to be diluted to 1:80 to reduce this anticomplementary action sufficiently for use with horse complement. Despite this the C.C.A.T. with the antigen at 1:80 detected antibody to a higher titre than the H.C.F.T. with the antigen at 1:4 (Fig. 1). But since the optimal antigen dilution with the H.C.F.T. was 1:4 it seemed probable that still higher titres would be obtained in the C.C.A.T. if the antigen could be rendered less anticomplementary and used in higher concentration. For this purpose the following methods were investigated.

I. *Chloroform extraction in the aqueous phase.*—A method of chloroform treatment was successful in removing the anticomplementary effect of the influenza mouse lung antigen (see below) and this was also attempted with L.G.V. antigen. For influenza, the chloroform treatment was combined with centrifugation at 5000 r.p.m. for 15 minutes in an angle centrifuge. Unlike influenza, however, the main complement-fixing antigen of L.G.V. is associated with the larger particle elementary body and lower speed centrifugation at 2000 r.p.m. for 5 minutes was therefore used. (In the absence of chloroform, centrifugation at this speed did not affect the antigenicity). This method of chloroform extraction of the " wet " antigen effectively removed all the anticomplementary effect of the antigen but it also removed the specific antigenicity completely and it was therefore discarded.

II. *Extraction in the dried state by various solvents.*—De Boer and Cox (1947) have studied the extraction of brain and yolk sac antigens in the dried state by various fat solvents. They showed that benzene was most successful in removing the anticomplementary activity of the antigens for guinea-pig complement and also for reducing the non-specific fixation that occurs with human syphilitic sera. A modification of this technique was applied to the L.G.V. antigen in an attempt to remove the anticomplementary activity for horse complement.

Benzene, ether or chloroform were added to separate batches of freeze-dried L.G.V. antigen to twice the original volume. After extraction for one hour at room temperature the solvents were removed. Fresh solvent was added and the process of extraction repeated twice. The residual solvents were removed in various ways. Ether was evacuated under reduced pressure at room temperature. Benzene was removed either by this method, or by three rapid washes with ether which was itself removed under reduced pressure. Chloroform was also removed by rapid washing with ether and final drying in the same way.

These extracted antigens were then reconstituted in distilled water. Considerable difficulty was found in resuspending the extracted antigen evenly while antigen which had been dried but not extracted resuspended perfectly well. The final preparations were then tested for anticomplementary and antigenic activity by titration in serial dilution in the presence of two units of horse or guinea-pig complement with or without human L.G.V. antiserum. The results are shown in Table I. It will be seen that whenever a solvent was used the anticomplementary activity for horse complement was reduced. When this occurred, however, the antigenicity was also lowered, usually to a corresponding degree.

These methods of treating antigens with fat solvents were successful for the influenza and vaccinia systems (see below) but were discarded in the case of the L.G.V. antigen.

III. *Complement treatment of antigen.*—It seemed possible that the method used by Taran (1946) to remove the anticomplementary action of certain human sera for guinea-pig complement might be modified so as to remove the anticomplementary action of the L.G.V. antigen for horse complement. Taran's method consists of treating the serum with an excess of guinea-pig complement and subsequently inactivating any free complement by heating at 56° C. for 30 minutes.

Obviously this method can only be applied to antigens which are unaffected

TABLE I.—*Titration of L.G.V. Antigen Treated by Various Methods, in the Presence of 2 M.C.D. of Horse and Guinea-pig Complement to show Anti-complementary Action, also with L.G.V. Human Antiserum to show Anti-genicity.*

Other reagents.	L.G.V. antigen. Method of treatment.	Antigen dilutions. C.C.A.T. 2	4	8	16	32	64	128	256	H.C.F.T. 2	4	8	16	32	64
2 M.C.D. complement and saline	Untreated	0	0	0	0	4	4	4	4	4	4	4	4	4	4
	Dried unextracted	0	0	0	0	3	4	4	4	4	4	4	4	4	4
	Dried ether extracted	0	0	4	4	4	4	4	4	—	—	—	—	—	—
	Dried benzene extracted	0	0	4	4	4	4	4	4	—	—	—	—	—	—
	Dried benzene-ether extracted	0	0	4	4	4	4	4	4	4	4	4	4	4	4
	Dried chloroform-ether extracted	0	1	4	4	4	4	4	4	4	4	4	4	4	4
	Complement treated	4	4	4	4	4	4	4	4	4	4	4	4	4	4
2 M.C.D. complement and L.G.V. human antiserum 1:64	Untreated	0	0	0	0	0	0	0	4	0	0	0	2	4	4
	Dried unextracted	0	0	0	0	0	0	0	4	0	0	0	0	4	4
	Dried ether extracted	0	0	0	0	0	4	4	4	—	—	—	—	—	—
	Dried benzene extracted	0	0	0	0	2	4	4	4	—	—	—	—	—	—
	Dried benzene-ether extracted	0	0	0	4	4	4	4	4	4	4	4	4	4	4
	Dried chloroform-ether extracted	0	0	0	0	4	4	4	4	4	4	4	4	4	4
	Complement treated	0	0	0	0	0	0	0	4	0	0	0	2	4	4

4 indicates complete haemolysis or conglutination.
3, 2 and 1 indicate degrees of partial haemolysis or conglutination.
0 indicates complete inhibition of haemolysis or conglutination.

by a temperature of 56° C., but it seemed that it might be suitable for the L.G.V. antigen since it is very heat-stable. Preliminary experiments showed that treatment with a large excess of horse complement reduced the antigenicity of the antigen and it was therefore necessary to find the most suitable strength of complement to use. Constant amounts of antigen were treated with equal volumes of different dilutions of horse complement. After inactivation of any free complement the treated antigens were tested for their antigenic activity by checkerboard titrations with L.G.V. human antisera.

Five 5 ml. quantities of horse complement diluted 1:2·5, 1:5, 1:10, 1:20 and 1:40 respectively were added to equal volumes of L.G.V. antigen diluted 1:2; 5 ml. of saline were added to a sixth batch of antigen as a control. After standing for 2 hours at room temperature (18° C.) all the mixtures were heated to 56° C. for 30 minutes and then left at + 4° C. until the following day when the tests were performed. In the first place horse complement was titrated in the presence of various dilutions of each of the 6 treated antigens. Using the appropriate 2 M.C.D., a human L.G.V. antiserum was then tested with the dilutions of each antigen in checkerboard fashion using a fixation period of 30 minutes at room temperature. The antiserum was also tested by the H.C.F.T. using untreated antigen. The results are shown in Fig. 2 and Table 1.

This procedure certainly removed the anticomplementary effect as shown by the reduction in the amount of complement required to supply 2 M.C.D. in the presence of the treated antigens. Although treatment of the antigens with a large excess of horse complement (e.g. 1:2·5) reduced the antigenicity, a concentration of 1:10 or 1:20 removed most of the anticomplementary activity and left the antigenicity apparently unimpaired. Horse complement diluted

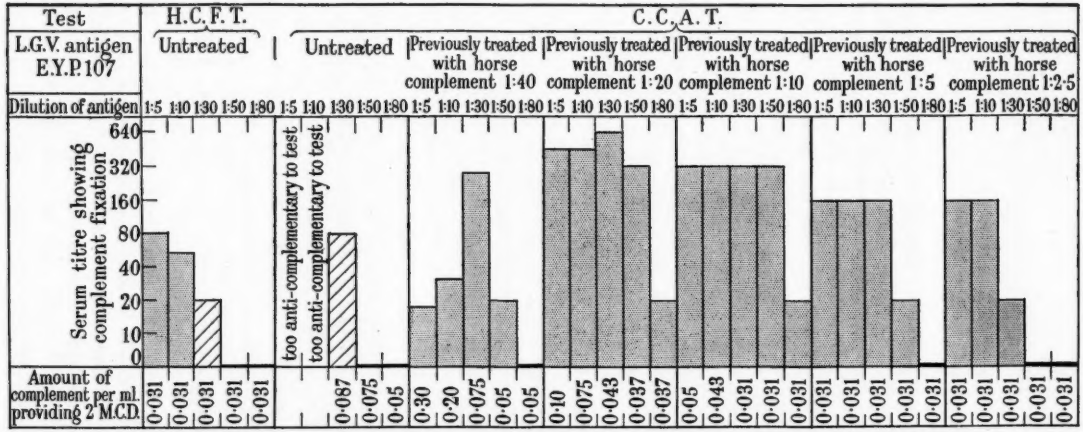

FIG. 2.—The effect of preliminary treatment of L.G.V. antigen with horse complement to remove the anti-complementary action of this antigen in the C.C.A.T.

The figure shows the results of checker-board titrations by the C.C.A.T. with a human L.G.V. serum and complement treated L.G.V. antigens. The five antigens compared were previously treated with an equal volume of horse complement diluted 1 : 2·5, 1 : 5, 1 : 10, 1 : 20 and 1 : 40 respectively and subsequently inactivated. Untreated control antigens were also examined by both the C.C.A.T. and H.C.F.T.

The dilution of horse complement containing 2 M.C.D. found in the presence of saline was ·031 per ml. ; the dilutions providing 2 M.C.D. in the presence of the various antigens are shown in the figure.

(The L.G.V. antigen used in this experiment is a different one to that used in the experiment described in Fig. 1 and is less powerful.)

1:10 or 1:15 was therefore used to treat the antigen (subsequently called complement-treated antigen) whenever this method for removal of anticomplementary activity was adopted.

The treatment is best carried out just before use of the antigen but the treated antigen is stable for at least 14 days at + 4° C. When vaccinia antigen was treated in this way the anticomplementary activity returned after several weeks at + 4° C. or — 20° C.

(b) *Vaccinia Antigen.*

The crude vaccinia antigen prepared as described was anticomplementary to horse complement when used at concentrations greater than 1:32. It was important to remove this effect because although with the antigen V14 the optimal antigen dilution was as high as 1:256–1:512, in the case of V11 and V12 the optimal dilution was only obtained using the antigens as concentrated as possible. Antigen V14 was prepared in the same way as V11 and V12 and it is not known why the antigenicity was so much greater.

I. *Benzene-ether extraction.*—On extracting the dried antigen with benzene and ether as described for the L.G.V. antigen, the anticomplementary effect against horse complement was completely removed while the antigenicity was left unaffected. This method also removed the slight anticomplementary effect against guinea-pig complement. These results are shown in Table II from which it may also be seen that mere freeze-drying and resuspension of the antigen removed much of the anticomplementary effect.

II. *Chloroform-ether extraction.*—Extraction of the dried vaccinia antigen with chloroform followed by ether in the same manner as described for the benzene extraction also completely removed the anticomplementary effect. This did not reduce the antigenicity as shown by the C.C.A.T. although a four-fold reduction in the antigen titre was shown by the H.C.F.T. These results are also shown in Table II.

TABLE II.—*Showing the Effect of Freeze-drying combined with Benzene-Ether or Chloroform Ether Extraction on the Anticomplementary Action and Antigenicity of Vaccinia Antigen.*

Other reagents.	Vaccinia antigen V14. Method of treatment.	Antigen dilutions. C.C.A.T. N	2	4	8	16	32	H.C.F.T. N	2	4	8	16
2 M.C.D. complement and saline	Untreated	0	0	0	0	2	4	3	3	4	4	4
	Dried unextracted	0	1	4	4	4	4	2	3	4	4	4
	Dried benzene-ether extracted	4	4	4	4	4	4	3	4	4	4	4
	Dried chloroform-ether extracted	4	4	4	4	4	4	4	4	4	4	4
		256	512	1024	2048	4096	8192	256	512	1024	2048	4096
2 M.C.D. complement and vaccinia rabbit antiserum 1:40	Untreated	0	0	0	0	4	4	0	0	0	1	4
	Dried unextracted	0	0	0	0	4	4	0	0	0	2	4
	Dried benzene-ether extracted	0	0	0	1	4	4	0	0	1	3	4
	Dried chloroform-ether extracted	0	0	0	1	4	4	0	0	3	4	4

4 indicates complete haemolysis or conglutination.
3, 2 and 1 indicate degrees of partial haemolysis or conglutination.
0 indicates complete inhibition of haemolysis or conglutination.

(c) *Influenza.*

Chloroform extraction in the aqueous phase.—This is the method of treatment used by Hoyle (1948) and recommended by the World Influenza Centre for the preparation of diagnostic antigen from mouse lungs in routine complement fixation tests for influenza. It removed completely the anticomplementary effect of the antigen for both horse and guinea-pig complement and it has the additional advantage of removing Forsmann antigen without affecting specific antigenicity (Hoyle, 1948). Since this method was successful no other was attempted.

The technique was as follows: Dried mouse lungs infected with influenza A (PR8 strain) were ground with sterile sand and made up to 1 per cent with saline. Sodium azide 0·08 per cent was added as preservative. After centrifuging at 5000 r.p.m. in the angle centrifuge for 15 minutes the supernatant was treated with 5 per cent chloroform, shaken vigourosly, allowed to stand overnight at + 2° C. and next day recentrifuged at 5000 r.p.m. for 15 minutes. If still anticomplementary the chloroform treatment and centrifugation were repeated. It was generally found that one treatment was sufficient to remove the anticomplementary action for guinea-pig complement and a further one or two treatments enough to remove the anticomplementary effect for horse complement. There was no apparent effect on antigenicity.

2. *Anticomplementary Action of Sera for Horse Complement.*

None of the rabbit sera used in this work was anticomplementary (at a dilution of 1:5 or greater) to guinea-pig or horse complement. This confirms previous experience that rabbit serum is not as a rule anticomplementary to horse complement.

The proportion of human antisera showing this effect against guinea-pig complement is well known. Of 30 human sera tested by the C.C.A.T. for antibodies to the L.G.V. antigen, only one was found to be anticomplementary to horse complement and then only at a dilution of 1:5. Of 32 specimens of human sera tested for vaccinia antibodies, 7 were anticomplementary to the 2 M.C.D. of horse complement but only one of these showed the effect at a dilution of over 1:10. However, a number of human sera collected over a wide area and sent for examination to the Virus Reference Laboratory of the Public Health Laboratory Service at Colindale contained a very high proportion which were anticomplementary to horse complement; in fact only 6 of the 19 were free of anticomplementary effect at 1:5. Five of the remaining 13 were anticomplementary even at a dilution of 1:40. Four of the series were also anticomplementary for guinea-pig complement. The reason for these results is not obvious since human serum in our experience is not usually anticomplementary to horse complement. It is possible that transmission by the post was responsible. One of the highly anticomplementary sera was preserved with sodium azide but experiments showed that the addition of this preservative to sera does not increase their anticomplementary effect for horse complement.

3. *Influence of Time and Temperature on Fixation of Complement.*

Earlier studies comparing the H.C.F.T. and C.C.A.T. were carried out with a 30-minute fixation at room temperature (Coombs and Hole, 1948). In later

studies comparisons were made using 30 minutes at room temperature and overnight at + 4° C. (Blomfield, Coombs and Hole, 1949). In most cases the results obtained by the H.C.F.T. were markedly improved by the overnight method but those with the C.C.A.T. were hardly improved at all. With virus antigens Bedson and B and (1929) have shown that overnight fixation at + 4° C. was often preferable for the H.C.F.T.

In our present experiments it was therefore necessary to find the optimal time and temperature for fixation for each system before a comparison of the relative merits of each test could be made. In all the experiments summarized here the complements were titrated in the presence of the antigens under the same conditions as in the test itself.

(a) L.G.V. System.

Using L.G.V. antigens and human antisera the H.C.F.T. and C.C.A.T. were compared using fixation under the following conditions: 30 minutes at room temperature, 2 hours at room temperature, 15 minutes at room temperature followed by $1\frac{3}{4}$ hours at + 4° C. and overnight at + 4° C. followed by 30 minutes to 1 hour at room temperature. Complement-treated antigen diluted 1:8 was used for the C.C.A.T. and untreated antigen at 1:4 for the H.C.F.T. The results are shown in Table III.

TABLE III.—*Influence of Conditions of Fixation on H.C.F.T. and C.C.A.T. in a L.G.V. Human Antiserum System.*

Conditions of fixation.	Serum titres.	
	H.C.F.T.	C.C.A.T.
30 minutes at room temperature	10	40
2 hours at room temperature	20	80
15 minutes at room temperature ; 105 min. at + 4° C. .	20	40
Overnight at + 4° C.	20	40

It will be seen that the serum titre for the C.C.A.T was maximal after fixation for 2 hours at room temperature. The fact that the overnight titre was lower may be due to the larger dose of horse complement necessary to supply 2 M.C.D. because of an increase in anticomplementary action of the antigen with overnight fixation. The 2-hour fixation period at room temperature was also one of the most satisfactory for the H.C.F.T. and so it was considered optimal for both systems.

(b) Vaccinia System.

Fig. 3 shows a comparison of room temperature fixation for 30 minutes with overnight fixation at + 4° C., using checkerboard titrations of benzene-extracted vaccinia antigen against rabbit antiserum. In addition to the H.C.F.T. with guinea-pig complement, the C.C.A.T. was performed with cat and pig as well as horse complements. These other conglutinating complements were included because the earlier work had suggested that in testing rabbit antisera horse complement had only a slight superiority over the other two.

For the short period of fixation horse complement was much more sensitive than guinea-pig complement, but in the overnight test the sensitivity of guinea-pig increased to that of horse complement and surpassed the cat and pig con-

glutinating complements. This is in agreement with the results obtained with the mallein system (Blomfield, Coombs and Hole, 1949).

A further comparison was made using the more potent vaccinia antigen V14; the antigen being used at a dilution of 1:128. In this experiment the following periods for fixation were compared: half an hour at room temperature, 2 hours at room temperature and overnight at + 4° C. The results are shown in Table IV This experiment confirmed that overnight fixation is optimal for the H.C.F.T., while in the case of the C.C.A.T. the results with the half hour and 2-hour fixation period at room temperature are as good as those obtained with the overnight fixation period.

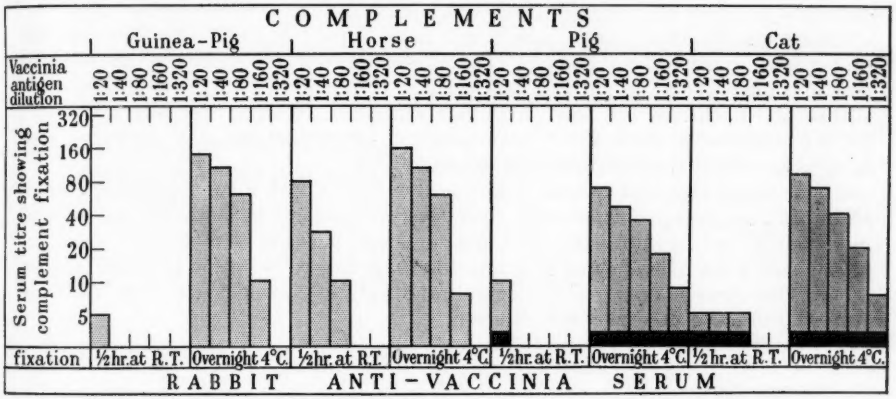

FIG. 3.—Influence of conditions of fixation on C.C.A.T. and H.C.F.T. The figure shows checkerboard titrations of a rabbit antiserum (VR10) with benzene-extracted vaccinia antigen (VII). Fixation periods of half an hour at room temperature (R.T.) and overnight at 4° C. are compared using the H.C.F.T. with guinea-pig complement and the C.C.A.T. with horse, pig and cat complements.

TABLE IV.—*Influence of Conditions of Fixation on the H.C.F.T. and C.C.A.T. in a Vaccinia Rabbit Antiserum System.*

Conditions of fixation.	Demonstrable antibody titres.	
	H.C.F.T.	C.C.A.T.
Half an hour at room temperature	0	160
Two hours at room temperature	20	160
Overnight at + 4° C.	80	160

(c) *Influenza System.*

This system differed from the L.G.V. in that overnight fixation gave greater sensitivity for the C.C.A.T. and the H.C.F.T. A pool of human influenza A antisera was titrated with influenza A antigen at 1 6, comparing fixation for 2 hours at room temperature and overnight at + 4° C. with both the H.C.F.T. and C.C.A.T.

By the H.C.F.T. the titre of the pooled serum after a 2-hour fixation was 1:80 and rose to 1:320 with the overnight test. The titre with the C.C.A.T.

which was 1:640 after a 2-hour fixation, rose to 1:1280 after overnight fixation. Overnight fixation at + 4° C. was therefore used in all tests with influenza antigen.

4. "*Normal*" *Antibodies.*

(*a*) *Rabbit Serum.*

It was only in the vaccinia system that rabbit antiserum was used. The vaccinia antigen if fairly concentrated, even after benzene extraction, gave a definite fixation of horse complement in the presence of normal rabbit sera at dilutions sometimes as high as 1:40 ; a partial fixation was also obtained with the H.C.F.T. although to a lower serum titre. Normal chorioallantoic membrane extract also gave this reaction although again only to a lower serum titre.

For example, two normal rabbit sera fixed horse complement in the presence of benzene extracted vaccinia (V14) antigen diluted 1:4 although no reaction was obtained when the antigen was used at a dilution of 1:128, a dilution well within the optimum for this particular antigen. Heating the sera to 65° C. for 30 minutes reduced this "non-specific" effect and did not alter the titre of specific antibody in the immune serum.

Thus it appears that normal rabbit serum reacts with a component in chorioallantoic membrane extract and that this component is increased in amount in virus-infected membranes. This phenomenon, whether in fact it is "specific" or not (see work of Kidd and Friedewald, 1942) has to be borne in mind in cases where the virus content of the membrane extract antigen is so low that the antigen has to be used at a low dilution.

(*b*) *Human Serum.*

Human sera were used in tests with all three virus systems investigated. The sera of 9 out of 13 unvaccinated apparently normal individuals fixed horse complement in the presence of low dilutions of either benzene-extracted, chloroform-extracted or complement-treated vaccinia antigens. The serum titres giving this reaction were between 1:20 and 1:80 and several sera actually showed an antiserum prozone effect. A similar reaction although to a lower serum titre was given with normal chorioallantoic membrane extract. These sera were all tested and found to be Wassermann negative. Heating the sera to 65° C., as with normal rabbit sera, reduced this effect.

As mentioned in the case of normal rabbit sera, this phenomenon could be due to a "naturally occurring" antibody in human serum reacting with a component in the membrane extract, a component which is increased in amount in infected membranes.

The fact that none of these sera showed the effect with the H.C.F.T. might well be explained by the fact, as already shown (Blomfield, Coombs and Hole, 1949), that horse complement is much more sensitive than guinea-pig complement in detecting certain antibodies in human sera.

"Non-specific" reactions have not been found with any normal human sera using untreated or complement-treated L.G.V. antigen. This is perhaps surprising as the antigen is derived from yolk sacs of fertile hens' eggs and a reaction was detected between normal egg chorioallantoic membrane and normal human serum as mentioned above. The fraction in normal egg responsible for

this reaction may, however, be heat labile and therefore destroyed during the preparation of L.G.V. antigen.

No reaction between chloroform-extracted influenza A mouse-lung antigen and three normal human sera has been detected by either the H.C.F.T. or C.C.A.T.

5. *Comparison of the H.C.F.T. and C.C.A.T. in the Three Virus Systems Using the Conditions Found to be Optimal for Each System.*

(*a*) *Vaccinia.*

Three rabbit antivaccinia sera were examined for their titre of complement-fixing antibodies using the benzene-ether extracted antigen V14 diluted 1:128 and a fixation period of overnight at + 4° C. The results are given in Table V. Five normal rabbit sera showed no fixation with either test.

TABLE V.—*Comparison of H.C.F.T. and C.C.A.T. in the Examination of Rabbit Antivaccinia Sera Using an Overnight Period of Fixation at + 4° C.*

Sera.	Serum titres.	
	H.C.F.T.	C.C.A.T.
Rabbit V10	80	160
„ 1287	80	160
„ 1290	1280	1280

No complement-fixing antibodies could be detected by either test in human sera up to a period of 30 days following successful vaccination of 10 individuals.

(*b*) *L.G.V.*

The sera were some which had been referred to the London Hospital for examination for either L.G.V. or psittacosis antibodies.

Complement-treated L.G.V. antigen was used in a dilution of 1:8 for the C.C.A.T., while for the H.C.F.T. untreated antigen diluted 1:4 was employed. Fixation was for 2 hours at room temperature which was optimal for both tests.

The results together with those obtained with the H.C.F.T. at the London Hospital are recorded in Table VI. With the exception of the sera from two patients, R.51 and R. F., the results with the two types of test are in agreement but where positive the titres given by the C.C.A.T. are generally higher. In the two exceptions the C.C.A.T. gave a positive finding while the result with the H.C.F.T. was doubtful (interpreted as negative).

The clinical condition of R. F. was not typical of L.G.V., and with the negative Frei test this supported the H.C.F.T. rather than the C.C.A.T. R.51 was more difficult clinically and the doubtful Frei test did not assist the diagnosis. It is therefore not possible to decide whether the H.C.F.T. or the C.C.A.T. gave the correct result.

The results in doubtful cases such as these are clearly of great importance in deciding the merits or otherwise of the new test. Unfortunately no final standard of reference exists except the isolation of the causal organism which is not often possible.

TABLE VI.—*Comparison of H.C.F.T. and C.C.A.T. in the Examination of Human Sera with L.G.V. Antigen Using a 2-hour Period of Fixation at Room Temperature.*

Patient.	Clinical diagnosis.	Serum titres. H.C.F.T. London Hospital.	H.C.F.T. Cambridge.	C.C.A.T.
G. E.	Atypical pneumonia: 1st day	<4	<5	10 Anticomp. to 1:5
	21st ,,	<4	<5	<5
	32nd ,,	<4	<5	<5
F.	Doubtful L.G.V. (rectal lesion) Frei test negative	<4	<5	<5
E.	Atypical pneumonia	<4	<5	<5
T.360	Doubtful L.G.V. (preputial ulcer) Frei test negative	<4	<5	<5
A. K.	Atypical pneumonia ? psittacosis	<4	<5	<5
E. L.	Atypical pneumonia ? psittacosis	4 partial	<5	<5
T. D.	Doubtful L.G.V. (bilateral inguinal adenitis)	<4	<5	<5
Ch.	Continued fever ? psittacosis	4 partial	10	<5
R.51	Doubtful L.G.V. (perianal abscess and rectal induration) Frei test doubtful	4 partial	5	40
R. F.	Doubtful L.G.V. (fistula in ano) Frei test negative	4 partial	5 partial	40
B.1357	Established L.G.V. Frei test positive	8	20	40
T.245	,, ,,	16	40	160
P.	,, ,,	128–256	320	320
V. W.	,, ,,	64	160–320	640
T.307	,, ,,	64	320	1280
An.	,, ,,	32	40	160
A. S. M.	Psittacosis	128–256	160–320	640

Antigen diluted 1:4 for the H.C.F.T.
Antigen treated with complement then used at 1:8 for the C.C.A.T.

(c) *Influenza.*

A direct comparison of the C.C.A.T. and H.C.F.T. in the influenza system was made with chloroform-extracted influenza A antigen and overnight fixation at + 4° C. A pool of convalescent human influenza A antiserum and specimens from two individuals with influenza A infection were tested under these conditions.

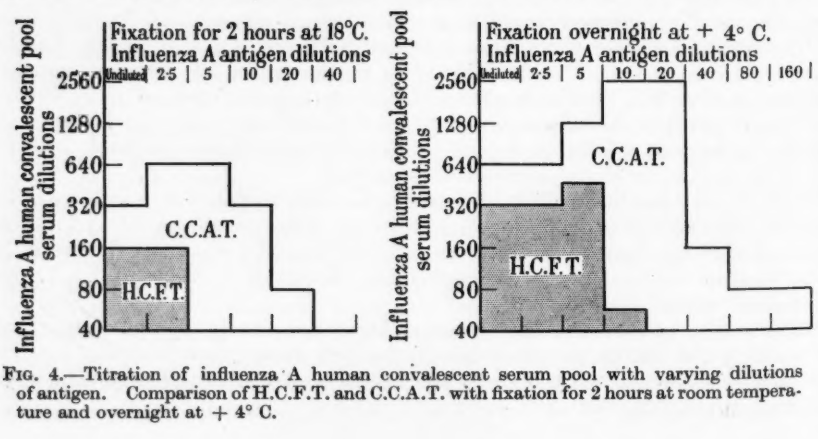

FIG. 4.—Titration of influenza A human convalescent serum pool with varying dilutions of antigen. Comparison of H.C.F.T. and C.C.A.T. with fixation for 2 hours at room temperature and overnight at + 4° C.

The results of a checkerboard titration of the convalescent influenza A serum pool in the presence of increasing dilutions of antigen are shown in Fig. 4. The optimal antigen dilution in the H.C.F.T. was 1:5 giving a serum titre of 1:320 (50 per cent. at 1:640). In the C.C.A.T. at this antigen dilution the serum titre was 1:1280 but it rose to 1:2560 when the antigen was further diluted to 1:10 or 1:20 while the H.C.F.T. titre fell sharply. The optimal antigen dilution is also influenced by the conditions of fixation as is shown by Fig. 4 where the result of the checkerboard titration made with fixation for two hours at room temperature is compared with the overnight method.

Of the specimens from individual cases of influenza, one gave a titre of 1:320 by the H.C.F.T. and 1:1280 by the C.C.A.T., and the other 1:320 by the H.C.F.T. and 1:2560 by the C.C.A.T. A specimen from the latter at the acute stage of the disease was negative by both tests. A more extensive comparison in influenza is described in another paper by Stoker and Marmion (1950).

DISCUSSION.

These experiments were undertaken to see if, as we expected, the C.C.A.T. would prove more sensitive than the H.C.F.T. in detecting the presence of virus antibodies, and at the same time to discover any factors which might limit the application of the C.C.A.T. due to the nature of the materials and method used. One problem which had to be overcome was the anticomplementary effect of crude virus tissue antigens against horse conglutinating complement. Extraction with certain solvents overcame the difficulty with vaccinia and influenza antigens but these methods led to a marked reduction in antigenicity when applied to L.G.V. antigen. The anticomplementary action of this antigen was removed by treatment with horse complement and subsequent inactivation, but we do not consider the method satisfactory for routine use. Treatment with too much complement led to a reduction in effective antigenicity, possibly due to the presence of the inactivated serum which in low dilutions tends to mask the specific fixation of complement (Blomfield, Coombs and Hole, 1950). In addition, treated antigens regained their anticomplementary effect on storage at + 4° C. or — 20° C.

A second problem was the apparent non-specific fixation of horse complement by some crude tissue antigens in the presence of normal sera. It is possible that this is due to naturally occurring antibodies reacting with a component in tissue extracts (Kidd and Friedewald, 1942) and is therefore not non-specific in the strictly serological sense. Greater sensitivity in the C.C.A.T. might reveal these reactions with consequent loss of specificity in the main system under investigation.

In direct comparisons of the two tests the results with rabbit antivaccinia sera are in agreement with previous observations on mallein and *Salmonella* rabbit antisera (Blomfield, Coombs and Hole, 1949, 1950). With human antisera, the C.C.A.T. did not show the uniformly increased sensitivity which had been anticipated from this previous work. Even so, serum and antigen titres were never less than those obtained by the H.C.F.T. and were often four or eight times higher.

It appears, therefore, that the C.C.A.T. can be used in the three virus systems which we have studied. Unfortunately, its greater sensitivity probably renders the test more susceptible than the H.C.F.T. to the non-specific effects often en-

countered with tissue antigens. Some of these difficulties have been overcome, but further investigation of the nature of the so-called non-specific reactions is an essential preliminary of the use of the C.C.A.T. in virus diseases.

SUMMARY.

The haemolytic complement fixation test and conglutinating complement absorption test have been compared in vaccinia, influenza and psittacosis-lymphogranuloma venereum antigen antiserum systems.

Crude virus antigens were highly anticomplementary for horse complement. Of the methods we investigated to overcome this difficulty, extraction of the influenza antigen with chloroform and benzene-ether extraction of the vaccinia antigen were the most successful. In the case of the L.G.V. antigen the method used consisted of treating the antigen with horse complement.

The presence of naturally occurring antibodies against virus and normal tissue antigens proved a complicating factor, especially in the conglutinating complement absorption test. The significance of these apparently "non-specific" effects is discussed.

The optimal conditions of fixation for each test in the three systems has been determined. In the influenza and vaccinia systems fixation overnight at + 4° C. was the most satisfactory for both tests, although for the conglutinating complement absorption test in vaccinia short periods of fixation gave almost as good results. For the L.G V. system two hours at room temperature was the optimal period for both tests.

Using these optimal conditions the sensitivities of the two tests have been compared. With rabbit antisera the sensitivity with the conglutinating complement absorption test was only slightly increased. With human antisera the titres demonstrated with this test were 2 to 8 times higher than those shown by the haemolytic complement fixation test. However, the advantage gained in increased sensitivity with the former test may be offset by the apparent "non-specific" reactions which have to be overcome.

We would like to express our indebtedness to Dr. R. G. White for the preparation of the four figures.

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QUANTITATIVE STUDIES IN DIPHTHERIA PROPHYLAXIS: THE SECOND RESPONSE.

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THE Second Response Phenomenon of Glenny and Südmerson (1921) showed that the response to a second (spaced) inoculation of under-neutralized diphtheria toxin-antitoxin-mixture is quite different from that following the first. The latent period following the first injection was some 3 weeks, as against 3 to 4 days following the second. The animals' sera attained their *maximum* antitoxic values some 3 to 8 weeks after the first inoculation, and in 8 to 12 days after the second. A further remarkable characteristic of the second response phenomenon is the great increase in circulating antitoxin as a consequence of very small secondary stimuli, such that, if given as primary stimuli, would induce undetectable amounts of circulating antitoxin (Glenny and Allen, 1922).

Recently, it has been shown that a pure primary response in guinea-pigs to an inoculation of diphtheria toxoid is one in which, after a latent period of some 14 days, antitoxin first appears in the animal's serum, rising to a maximum 11 to 14 days later, and remains substantially the same for 12 weeks ; in short, there is no peak value (Holt, 1949)

The subcutaneous inoculation into guinea-pigs of moderately large doses of adsorbed toxoid, such as A.P.T. or purified toxoid adsorbed on to $AlPO_4$ gel (P.T.A.P.), gives rise to a marked subcutaneous nodule. The antitoxin response to such an inoculation—used as a primary stimulus—is one with a marked peak value. It was shown that it was possible to obtain, by suitably timed excision of these nodules, an antitoxin response with no peak value (Holt, 1949).

It was concluded from these experiments that the peak character of the antitoxin response usually obtained from the inoculation of adsorbed toxoid represents a second response superimposed on a pure primary.

This was rendered all the more reasonable since it was also shown that a second response could be obtained as early as 10 to 14 days after primary inoculation. It follows, therefore, that a second response effect started 10 to 14 days after the primary inoculation, reached its maximum *at the same time* as that resulting from the pure primary response. Earliest time for maximum titre following a pure primary response is 25 to 28 days ; time for maximum response from a second stimulus is 12 to 14 days, therefore total time for the earliest superimposed second response effect is about 26 days after inoculation.

These results also demonstrated the apparent coincidence of time for the end

of the latent period for a primary response, and the earliest time to initiate a second response effect.

Once an animal has received a primary inoculation, and responded to it, as evidenced by the appearance of circulating antitoxin, the physiological situation has completely changed.

In keeping with the suggestion previously made (Holt, 1949) for the mechanism of the pure primary response, the state of affairs in the primed animal 10 to 12 weeks after its one inoculation is thought to be : (1) a number of normal globulin-producing cells, as a result of the original inoculation, have undergone a kainogenetic change, and continue to elaborate antitoxic instead of normal globulin, (2) many globulin-producing cells have undergone no such change, and (3) there is a uniform titre of circulating antitoxin resulting from the uniform production of antitoxin.

In respect of the antitoxin response to a delayed second inoculation of toxoid the position is complicated. An attempt has been made, however, to separate the several components quantitatively as follows in order to derive the character of a pure second response. The alterations in the serum antitoxin titres that follow a second (spaced) injection of toxoid would seem to be the resultant of three factors :

(1) the base-line level of circulating antitoxin obtaining before the second stimulus was given ;

(2) the pure second response or second response " leap," and

(3) the contribution towards the new base line level of circulating antitoxin brought about by the second inoculation.

It is only from certain types of second response curves, however, that the pure second response can be derived, as usually there is an elevation of the resting level of circulating antitoxin resulting from the second inoculation, which is disproportionately greater than would result from the same dosage used as a primary stimulus. This is a complication usually difficult to separate from the total effect in the early stages of the response, as the intermediate values are unknown, although the final result is evident from the elevated base-line level of circulating antitoxin.

It has proved possible, however, from the results obtained from recent experiments (designed with a totally different purpose) to derive the character of the pure second response effect.

In these experiments groups of 20 guinea-pigs were inoculated subcutaneously with 0·5 ml. of samples of P.T.A.P. containing 1 Lf/ml. of purified toxoid, but decreasing amounts of $AlPO_4$ carrier (pH 5 preparations). The antitoxin responses were followed for a period of 12 weeks.

The recipients of the preparation which produced the best response were divided into two equal subgroups, A and B. Subgroup A were then given a second identical inoculation of the same preparation of P.T.A.P., and Subgroup B 0·5 ml. of the preparation which produced the least primary response. The antitoxin responses were then followed over a further period of 12 weeks. The results obtained are shown in Tables I and II, and Fig. 1.

It will be seen from Tables I and II and Fig. 1 that when the best primary stimulus was used twice, the resting level of circulating antitoxin was doubled

as a result of the second inoculation. But when the primary stimulus was the most efficient, and the secondary stimulus was one very inefficient as a primary, there was no elevation of the pre-existing resting level of circulating antitoxin as a result of the second stimulus.

TABLE I.—*Serum Antitoxin Titres in Guinea-pigs which have received a Single Inoculation of P.T.A.P. of Varying Mineral Content.*

(Toxoid constant at 0·5 Lf injected.)

Results, average values in u/ml. serum.

Weeks after inoculation.	(i) Carrier injected 3 mg. $AlPO_4$.	(ii) Carrier injected 0·037 mg. $AlPO_4$.
2	0·07	<0·01
3	1·5	0·06
4	1·75	0·2
5	1·5	0·15
6	1·25	0·15
7	1·25	0·08
8	1·25	0·1
10	1·1	0·09
12	1·0	0·08
	(15 pigs)	(18 pigs)

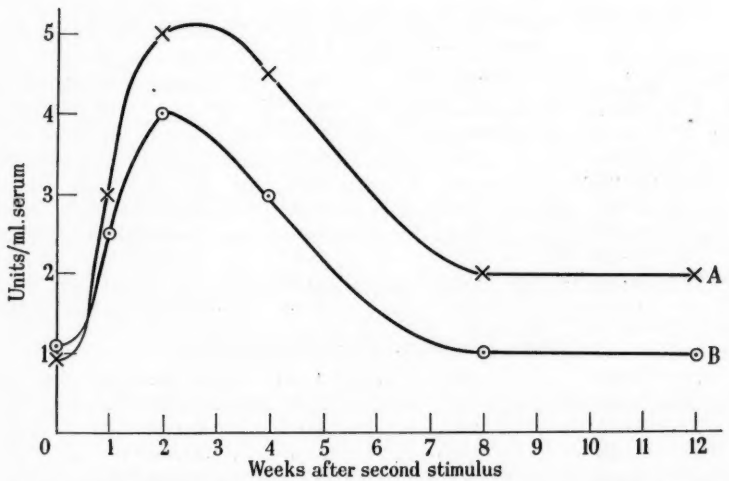

FIG.— 1.—Second responses in equally primed guinea-pigs to a second inoculation composed of

A. An excellent primary stimulus
B. A very poor primary stimulus
} (Table II).

TABLE II.—*Antitoxin Response in Primed Guinea-pigs (Group (i) in Table I) to a Second Stimulus*

Composed of

(*a*) 0·5 Lf toxoid with 3 mg. $AlPO_4$ carrier.
(*b*) 0·5 Lf toxoid with 0·037 mg. $AlPO_4$ carrier.

Time after 2nd injection (weeks).	Group A. Second stimulus 0·5 Lf, 3 mg. $AlPO_4$.	Group B. Second stimulus 0·5 Lf, 0·037 mg. $AlPO_4$.
0	0·95	1·05
1	3·0	2·5
2	5·0	4
3	(5·0)	(3·5)
4	4·5	3·0
6	(3·0)	(1·7)
8	2·0	1·0
12	1·9	1·0

(Numbers in brackets derived from the others drawn graphically.)

As the primary response curves to inoculations of both samples of P.T.A.P. had been obtained, it was possible to calculate in *both* cases the character of the pure second response effect.

This was done by subtracting from the total antitoxin response following the second inoculation, the initial resting level of circulating antitoxin plus the antitoxin titre attributable to the second inoculation acting as a primary stimulus; this of course, at the appropriate time intervals.

The data obtained by this procedure are shown in Table III, and graphically in Fig. 2.

TABLE III.—*Derived Pure Second Response (u/ml Serum).*

Weeks after second stimulus.	Base line titre.	Contribution of second stimulus acting as a primary.*	Total secondary response.†	Difference—the derived pure secondary.
Series A.—(3 mg. + 3 mg. $AlPO_4$.)				
0	0·95	*nil*	0·95	.. *nil*
1	0·95	,,	3·0	3–0·95 2·05
2	0·95	0·07	5·0	5–1·02 3·88
3	0·95	1·5	5·0	5–2·45 2·55
4	0·95	1·75	4·5	4·5–2·7 1·8
6	0·95	1·25	3·0	3–2·2 0·8
8	0·95	1·2	2·0	2–2·15 —0·15
Series B.—(3 mg. + 0·037 mg. $AlPO_4$.)				
0	1·05	*nil*	1·05	.. *nil*
1	1·05	,,	2·5	2·5–1·05 1·45
2	1·05	<0·01	4·0	4·0–1·05 2·95
3	1·05	0·06	3·5	3·5–1·1 2·4
4	1·05	0·2	3·0	3·0–1·25 1·75
6	1·05	0·15	1·7	1·7–1·2 0·5
8	1·05	0·1	1·0	1·0–1·15 —0·15

* Taken from Table I. † Taken from Table II.

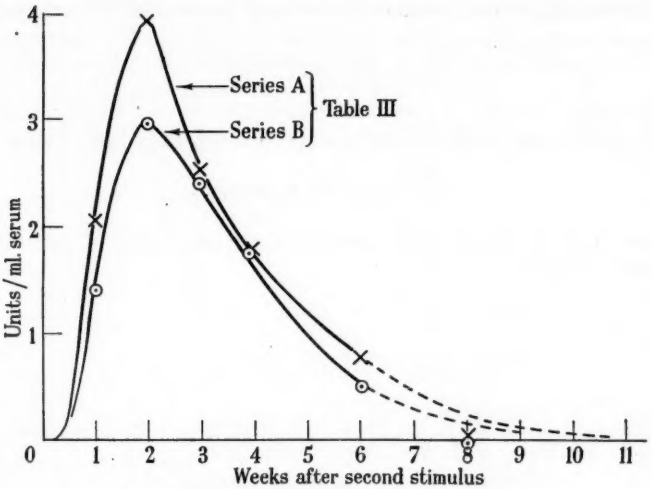

FIG. 2.—Derived pure second response or " leap."

Since it has been shown by Schoenheimer, Ratner, Rittenberg and Heidelberger (1942), using isotopic nitrogen, that the " half-life " of a globulin molecule is about 14 days, it should be possible to correlate their findings with the " negative " side of the derived pure second response effect. The rate of disappearance of the labelled protein in Schoenheimer's experiments showed it to be one in which the daily decrease was proportional to the amount initially present, and was therefore logarithmic in character.

The derived pure second response figures, shown in Table III, Column 5, were therefore plotted graphically, viz. log titre against time. The two graphs obtained are shown in Fig. 3. The plotted points from the Series 3 + 3 mg. gave an excellent straight line conformity ; those from the Series 3 + 0·037 mg. gave a less perfect conformity, but a straight line could be drawn within the limits of accuracy of the methods of antitoxin assay. It should be borne in mind that these figures are differences *between* two antitoxin determinations in every case, and the accuracy of assay is less the lower the titre to be measured.

Since the decline of titre is logarithmic, the mathematical constant may be derived from the equation :

$$\log T_m - \log T_2 = K.t,$$

where T_m = maximal titre,
T_2 = any intermediate titre at time t, in days ; whence from the 3 + 3 mg. curve,

$$K = \frac{\log 3{\cdot}88 - \log 1{\cdot}8}{14} = 0{\cdot}0238,$$

K for a 50% decline in 14 days = 0·0215.

Alternately the daily rate of decline of titre may be derived from the equation :

$$T_2 = T_m \left(1 - \frac{r}{100}\right)^t$$

where T_2, T_m, and t are as above, and r = percentage daily loss of titre.

r is found to be 6 per cent,

namely 6 per cent of the antitoxin present at the beginning of the day has disappeared after 24 hours.

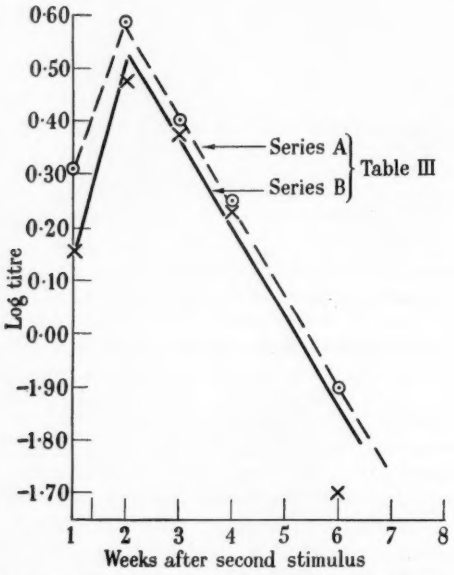

FIG. 3.—Graph ; log titre second response " leap "/time after second stimulus.

The same argument and method of analysis was then applied to data already described under the title " The Primary Response " (Holt, 1949), where the subcutaneous nodules were excised at different time intervals from animals which had received an inoculation of purified toxoid adsorbed on to $AlPO_4$ gel. This enabled an analysis to be made of data derived from observations made on animals of the same age, and in which (*a*) the two responses—both primary and secondary—were taking place simultaneously, and (*b*) a primary response only was taking place. This had the additional advantage that there was no initial or base-line level of immunity to allow for, and this greatly increased the accuracy of the differences of titre.

The titration figures together with the calculations are shown in Table IV, and the graph, log difference of titre against time, in Fig. 4. It will be seen that

TABLE IV.—*Derived Character of Pure Second Response.*

(Using data from experiments where the subcutaneous nodules were excised 7 days after inoculation of P.T.A.P., Holt, 1949.)

Time after inoculation (weeks).	Titre in animals' nodules excised 14 days or later after injection (u/ml. serum).	Titre in animals' nodules excised 7 days after injection (u/ml. serum).	Difference in titre (u/ml. serum).
2	0·2	0·2	0·0
3	1·5	0·4	1·1
4	2·5	0·75	1·75
5	1·6	0·6	1·0
6	1·25	0·6	0·65
8	0·9	0·6	0·3
10	0·7	0·6	0·1

$$K = \frac{\log T_m - \log T_2}{t} = 0{\cdot}0295.$$

Percentage daily loss of titre r from $T_2 = T_m \left(1 - \frac{r}{100}\right)^t = 7.$

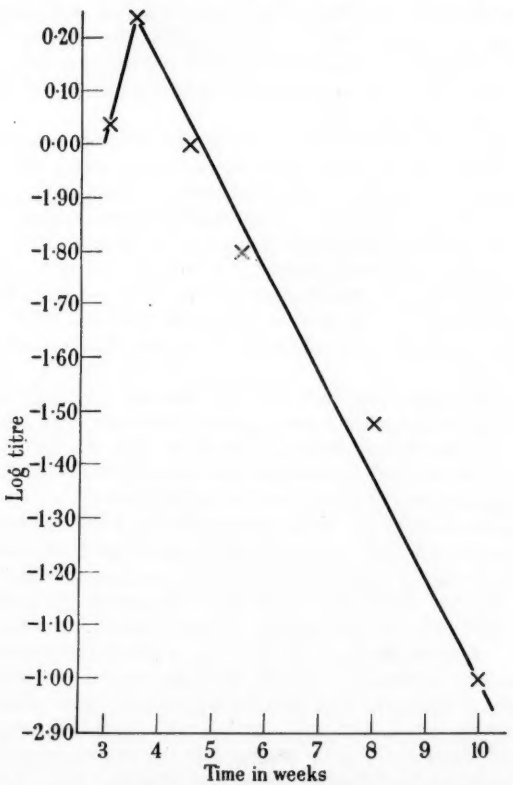

FIG. 4.—Graph ; log derived second response titre/time (Table IV).

very good conformity is obtained. The constant $K = T_m - T_2/t$ in this case was a little higher than previously obtained, being $K = 0{\cdot}0295$, and the daily decrease in titre rather greater (7 per cent.) These are probably attributable to the fact that the animals used in the latter experiment were three months younger than the others, and were diluting their antitoxin by the natural increase in plasma volume occurring with growth.

DISCUSSION.

The above evidence is considered to show clearly that the second response phenomenon may be reduced to three separate components: the initial titre of serum antitoxin, the antitoxin "leap" or the pure second response effect, and a third and most complicated phenomenon, the elevation of the resting level of circulating antitoxin. In this present communication consideration is restricted to the second response "leap" *per se*.

It has now been shown, using two quite different types of experiment, that the decline in the titre of the second response leap from its maximum is logarithmic. It had already been shown that the *ascent* to maximum was logarithmic (Burnet, Freeman, Jackson and Lush, 1941). The logarithmic decline in antibody titre is a characteristic of passive homologous immunity (Glenny and Hopkins, 1923), and has recently been shown to hold true for passive immunity of maternal origin in children (Barr and Glenny, 1949).

The agreement in character and degree of the decline in titre observed in the second response leap—or derived pure second response—with that found for passive (homologous) immunity and the isotopic nitrogen in the globulins of animals fed with isotopic nitrogen amino acids would appear most significant. In the examples of passive immunity there can be no possibility of a continuous production of antibody, and in the case of the isotopic nitrogen in plasma globulins, there was no evidence of a reutilization of the isotopic nitrogen from the disappearing plasma protein. All this is taken as evidence that once the second response leap has reached its maximum titre, no further release of antibody occurs from that cause.

Since, again, the time required for the second response leap to reach its maximum value lies within the latent period for a primary response, it is concluded that all the antitoxin showing itself as the second response leap was already present in the animal as reserve or stored antibody. This interpretation of the second response leap is being further investigated.

Further evidence in support of the suggestion that the second response leap is the release of reserve or stored antibody (globulin) has been described elsewhere (Holt, 1950). In brief it was found (1) that continuous daily stimulation of primed animals did not result in a change in the shape or timing of the second response curve, but only in the height of the maximum titre attained; and (2) that animals were relatively refractory to a third inoculation 40 days after a powerful secondary stimulus. These findings were interpreted as indicating (*a*) that a reserve of antibody had become exhausted, and therefore the animals were unable to respond to subsequent stimuli applied during that phase of exhaustion, and (*b*) that those cells responsible for the reserve would continue to make antitoxin and gradually build up a new reserve.

SUMMARY.

The Second Response Phenomenon of Glenny and Südmerson is examined. It is concluded that this phenomenon is the resultant of three separate components : (*a*) the initial serum titre, (*b*) the pure second response effect or " leap," and (*c*) the contribution of the second stimulus towards the new base-line level of immunity.

The decline in titre from maximum of the second response " leap " is logarithmic in character.

It is suggested that the second response " leap " represents the release of stored or reserve antibody, and as such is therefore not unlimited.

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THE IRIDESCENT PHENOMENON OF *PS. PYOCYANEA*.

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At least one half of freshly isolated strains of *Ps. pyocyanea*, cultured on solid media, show, when illuminated obliquely, a peculiar metallic or iridescent sheen (Jordan, 1899; Canzik, 1923), which may be disposed in discrete, irregularly shaped or nearly circular patches from 1 to 5 mm. in diameter (Fig. 1). In places, especially where bacterial growth is dense, these may be confluent, forming irregular areas of iridescence. Not infrequently a confluent growth of *Ps. pyocyanea* may be surrounded by an iridescent halo up to 5 mm. wide (Fig. 2), which may encroach on the growth in small wedge-shaped areas with the same shining appearance. The latter phenomenon is less common, and frequently occurs in subcultures from strains of *Ps. pyocyanea* which have been kept on a culture medium for some time.

These two appearances may occur independently or together, but there can be very little doubt that they are different expressions of the same phenomenon. However, iridescence or iridescent patches will here refer, unless otherwise stated, to the first, more common appearance. If a plate showing either of the two types of appearance is examined by transmitted light, the iridescent patches or haloes are seen to overlie areas where bacterial growth is absent or very much diminished (Fig. 1 and 2). These areas of diminished growth are frequently more nearly circular than the overlying areas of iridescence, and not infrequently there is a central papilla of what is apparently bacterial growth (Fig. 1). Some of the smallest patches resemble the plaques which are produced by the bacteriophages of *Ps. pyocyanea* when viewed by transmitted light (Fig. 3 and 4).

These appearances were described by Blanc (1923), Quiroga (1923), Hauduroy and Peyre (1923), and in greater detail by Hadley (1924), who showed quite clearly that the iridescent patches spread over the culture, and in doing so were associated with the disappearance of the bacterial growth underlying the spreading iridescence, a fact which has subsequently been confirmed.

A few communications by other workers on the subject of iridescent clearings in *Ps. pyocyanea* have been encountered in the available literature, and their conclusions can be summarized, together with those quoted, as follows: There are two schools of thought: firstly there is that which maintains that the iridescent phenomenon seen in *Ps. pyocyanea* is caused by bacteriophage because lytic areas occur in cultures on solid media, some of which superficially resemble bacteriophage plaques, and because typical bacteriophages can sometimes be isolated from filtrates of cultures of the organism which show the iridescent

phenomenon; secondly, there is that which holds the opposite view on the grounds that iridescent clearings frequently differ in appearance from bacteriophage plaques, and that all iridescent cultures will not yield bacteriophages, whereas some strains of *Ps. pyocyanea* not showing the iridescent phenomenon will do so.

It is obviously important to attempt to determine the nature of the iridescent clearings that occur in cultures of *Ps. pyocyanea* because, if they are due to bacteriophage, they present a singular example of the host-virus relationship, and a convenient field of study of a bacterial virus with unusual properties. If, on the other hand, the iridescent phenomenon is not due to bacteriophage, it is equally important to establish this fact because, firstly, workers will not be tempted to use it as a convenient means of investigating bacteriophage and the host-virus relationship and, secondly, a study of the phenomenon may reveal facts about the metabolism of *Ps. pyocyanea* of interest in themselves.

Thus, the purpose of this investigation is clearly to determine whether the iridescent clearings are due to bacteriophage action or not.

The work described in this paper is arranged under two main headings, "Descriptive" and "Experimental." In the first the important visible properties of the iridescent clearings are described and contrasted with bacteriophage plaques. In the second section experiments are described together with their results.

DESCRIPTIVE.

The iridescent clearings have been observed by the present author throughout the investigations described in this paper, and also during a period of three and a half years in routine hospital laboratories in which a large number of strains of *Ps. pyocyanea* have been encountered. During this time no observation has been made, concerning the appearance or general behaviour of iridescent clearings occurring in surface cultures of *Ps. pyocyanea*, which can be added to the detailed description of Hadley (1924), whose observations have been amply confirmed by numerous other workers. A general description of the iridescent phenomenon has been provided in the introduction to this paper, therefore only the important features that have been observed will be summarized here.

Iridescence was observed in some degree or other in 33 out of 65 strains of *Ps. pyocyanea*. It was most frequently associated with those cultures which produced the blue-green diffusible pigment, pyocyanin, in abundance, but this was not invariable. One strain (No. 101) which was practically yellow showed the phenomenon to a marked degree.

In routine hospital bacteriology most cultures of freshly isolated strains of *Ps. pyocyanea*, after incubation overnight, plated out in the usual way on common laboratory media, showed the iridescent phenomenon which consisted of either confluent irregular areas of iridescence or of iridescent patches. The patches varied in size from 1 to 5 mm.; moreover if such cultures were examined carefully, pin-point flecks of iridescence might be noted here and there, sometimes situated on single colonies. Infrequently there would be an iridescent halo, as described in the introduction (Fig. 2), surrounding areas of confluent growth of organisms. If such plates were incubated for a further 18 to 24 hours the iridescence as a whole could be seen to have spread, provided the plate was sufficiently moist; the pin-point flecks of iridescence had also spread with a marked increase in size,

and was accompanied by lysis of the bacterial growth in the area over which spread had occurred. New iridescent patches or pin-point flecks might also have appeared. After 48 hours' incubation, sometimes earlier, in nearly every case examined, crystals (Fig. 5) appeared which were just visible to the naked eye and were just beneath the surface of the agar, usually underneath the iridescence.

In these cultures, among the iridescent clearings, there were, not infrequently, other clearings resembling the former closely, but some of them did not show any iridescence at all, some showed striae of iridescence radiating from the centre of the clearings, and also other forms intermediate between these and the iridescent clearings proper. However when such cultures were incubated further every single one of these spontaneous clearings would become completely covered with iridescence.

The crystals mentioned above were examined more closely, and when observed with the $\frac{2}{3}$" objective of the microscope were seen to be arranged in sheaves, usually disposed radially, and lay in groups just beneath the upper surface of the agar (Fig. 5). If the cultures with which they were associated showed discrete iridescent clearings, the groups of crystals were found only at the base of the clearings and nowhere else, whereas if the culture showed confluent iridescent clearings, or there was a halo of iridescence or the plate was a particularly old one (four days or more), the groups of crystals were found to be distributed in other arrangements or haphazardly.

The visible properties of the iridescent clearings of *Ps. pyocyanea* which have been observed here are summarized and contrasted with those of bacteriophage plaques described previously (Warner, 1950) in Table I, from which it will be seen that the iridescent clearings were usually larger, very variable in size from day to day on the same medium, and less well defined than bacteriophage plaques, whereas the latter was smaller, more uniform in size from day to day, more perfectly circular and well defined. Iridescent clearings appeared in some cultures at any time up to 36 hours, and it could be seen that those that had appeared previously had spread considerably when re-incubated. Bacteriophage plaques appeared within a space of a few hours (less than 16), when growth of the organism was sufficiently opaque to form a background against which they could be seen after their first appearance; bacteriophage plaques showed no

EXPLANATION OF PLATE.

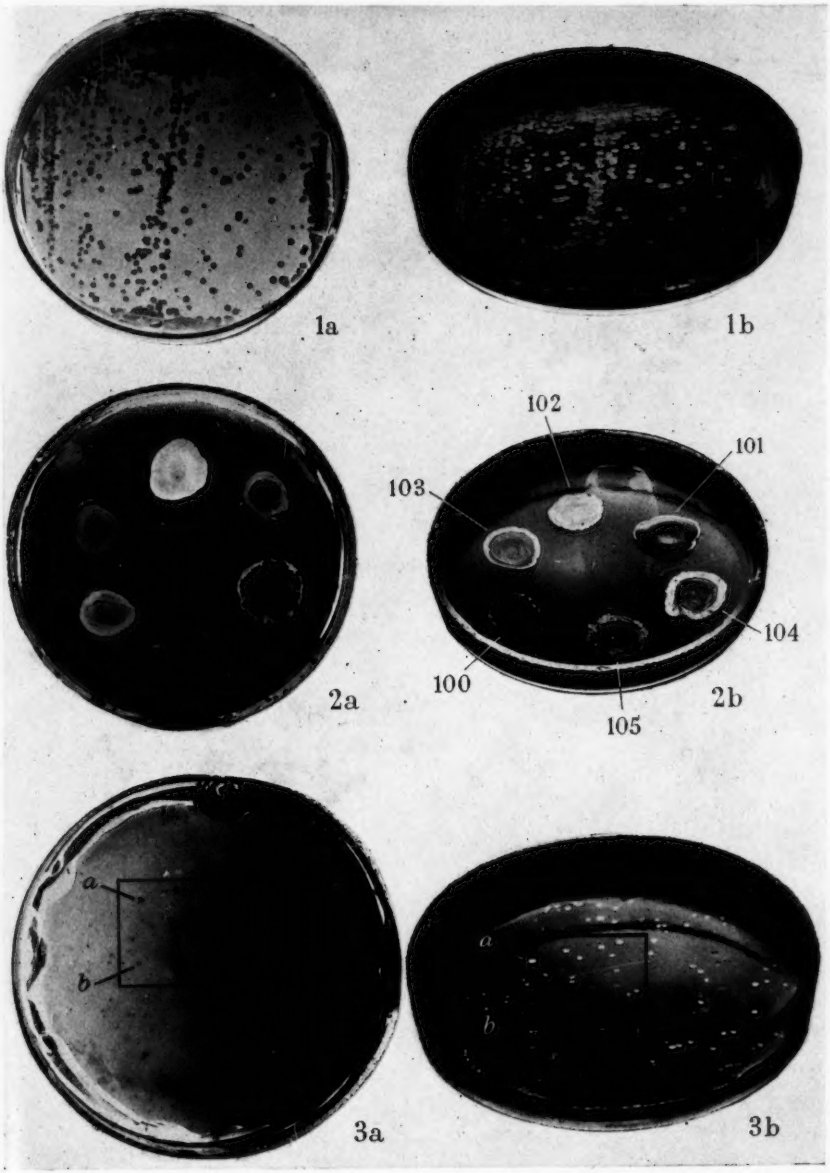
FIG. 1.—Shows iridescent clearings in a spread culture of Strain 1. In the photograph on the left the culture is viewed by transmitted light against a black background, and in the right by oblique illumination. Note in the former the nearly circular areas where bacterial growth is absent, and in which small central papillae are to be distinguished. On comparing the photographs on the left and right the clearings and the iridescent patches are seen to correspond exactly.

FIG. 2.—Cultures of *Ps. pyocyanea* No. 100, 101, 102, 103, 104 and 105 plated out to give a confluent growth. Strains 102, 103 and 104 clearly show the iridescent halo referred to in the text and the apparent lack of bacterial growth underlying it. The photograph on the left is viewed by transmitted light and the right by oblique illumination.

FIG. 3.—Shows a spread culture of *Ps. pyocyanea* No. 1 mixed with a suitable concentration of bacteriophage preparation 53/1. A is viewed by transmitted light and (b) by oblique illumination. Note that in (a) some of the iridescent clearings resemble bacteriophage plaques. The area enclosed within the lines is shown enlarged in Fig. 4—*a* and *b* are the same iridescent clearing and bacteriophage plaque in each case.

FIG. 4.—Enlargements from Fig. 3.

FIG. 5.—Shows the crystals often seen in cultures of *Ps. pyocyanea*. This photograph was taken of a culture of Strain No. 101 (about × 40).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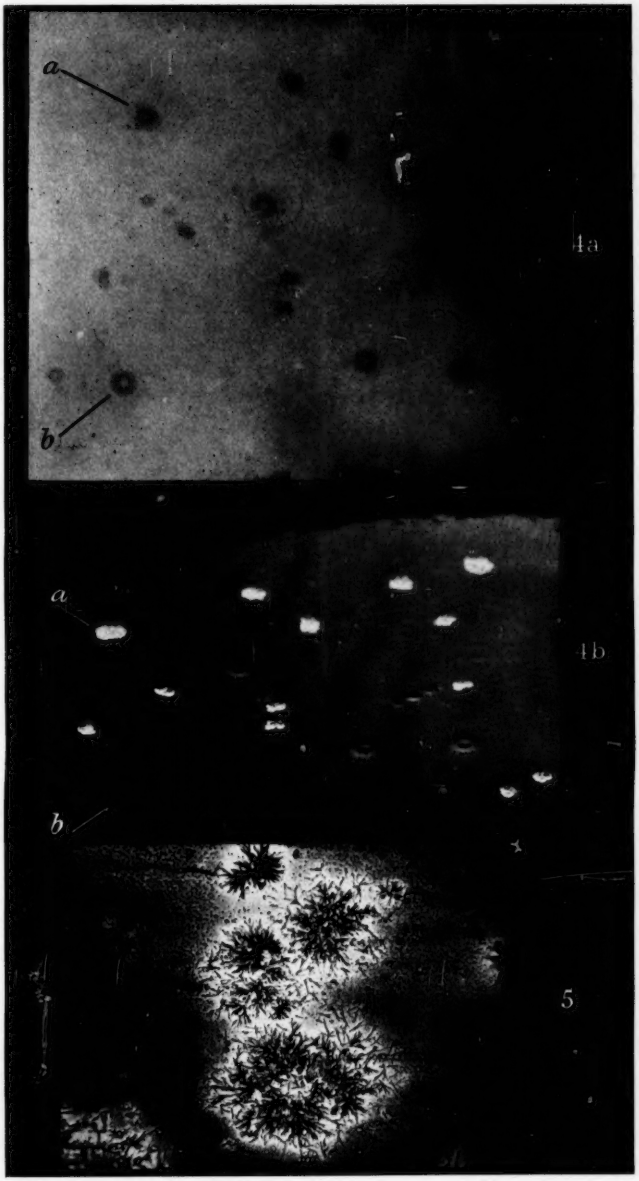

Warner.

TABLE I.—*Summary of Visible Properties of Iridescent Clearings and Bacteriophage Plaques.*

Bacteriophage plaques.	Iridescent clearings.
Uniform size on same medium	Size variable from day to day on same medium.
Usually smaller than clearings	Larger than plaques.
Well defined and circular	Less well defined. Not perfectly circular.
All appear within the space of a few hours (less than 16)	May appear at any time up to 36 hours.
Do not increase in size	Increase in size.
Never covered with iridescence	Always covered with iridescence.
Never associated with crystals	Nearly always associated with crystals.

appreciable increase in size. Finally, of the bacteriophage plaques examined previously (Warner, 1950) none was either covered with iridescence or associated with crystals, whereas all the spontaneous clearings which appeared in *Ps. pyocyanea* eventually became covered with iridescence within 48 hours and, very commonly, were associated with crystals just below the surface of the medium. In addition to the facts noted in Table I, bacteriophage activity has never been associated with an appearance which could be compared with haloes of iridescence (Fig. 2).

Separation of the Iridescent Material.

When observing a culture of *Ps. pyocyanea* showing the iridescent phenomenon to a marked degree, some water of condensation fell on to its surface. It was noted that the iridescent material was separated from the surface of the culture and floated on the water.

The iridescent patches appeared as thin, flat, irregularly shaped pieces of material with ragged edges when floated on water. These pieces usually kept the same shape as they had when on the culture that produced them. The pieces still showed iridescence although separated from the culture, and could be broken up quite easily by agitating the water on which they were floating. At the same time it was discovered that if chloroform were poured on to a surface culture of an organism showing iridescence, the iridescent material was dissolved.

As a result of these observations cultures were prepared of Strains No. 1 and 8 by spreading on nutrient agar (1·4 per cent) in 4½-in. Petri dishes, six plates being used for each strain. These were incubated for 48 hours. After incubation the cultures had produced abundant iridescent material. Sterile distilled water was poured on each plate, when the iridescent material was seen to float on the surface, imparting an iridescent appearance to it. The water and iridescent material from each plate was then poured into a separating funnel and the combined fluids were allowed to stand for a minute or two, when it was observed that the material with its characteristic appearance collected on the surface. Most of the water was then run out of the separating funnel from underneath. A fresh quantity of sterile distilled water was added, allowed to stand, and then the process repeated until the water appeared clear, and the iridescent material

was therefore thought to be relatively free of bacteria and any other unwanted material that happened to have been washed off the cultures. Most of the iridescent material was still floating on the surface, showing its characteristic appearance. Chloroform was added to the clear water and the two fluids shaken together, and allowed to stand until the chloroform and the water had separated into two layers. The chloroform layer was removed, placed in a test tube and evaporated by gentle heating.

The product obtained by evaporating the chloroform was a yellowish or brownish grey in colour, of a waxy consistency, and could be moulded with a glass rod; when pressed on paper it left what was apparently a greasy mark.

Since the amount of material was so small, and because the physical properties suggested a profitable line of investigation, described later, no chemical or biological investigation was performed.

EXPERIMENTAL.

The Inhibition of Iridescence in Cultures of Ps. pyocyanea.

Experiments are described here the aim of which was to inhibit the production of iridescence in surface cultures of *Ps. pyocyanea* by varying the conditions of atmosphere and temperature in which the latter were grown, and also by incorporating, in the nutrient medium used, a number of substances, including phenol, proflavine, organic and inorganic salts. In the course of these investigations it was observed that iridescence produced by strains of *Ps. pyocyanea* was inhibited by diminishing the amount of broth usually added to the agar media on which they were grown. This finding is also described here.

More important, however, was the fact that when the waxy and fatty or oily nature of the iridescent material was appreciated, the effect of surface active agents was tried on the iridescent material. This material was thought to have the properties which should be profoundly affected by such agents. Experiments were carried out in which this assumption was found to be correct, the results of which are described here.

In some of those cases where iridescence was inhibited by incorporating salts or surface active agents, or by diminishing the amount of broth in the nutrient media in use, bacteriophage activity was examined on the same media.

Material and Methods.

The strains of *Ps. pyocyanea* used were taken from those mentioned in "Descriptive," and the bacteriophage preparations were the same as previously described (Warner, 1950), except for one which was supplied by Dr. Dickinson.

In the first instance ordinary nutrient broth agar (2 per cent) slopes were used on which organisms of the various strains under test were grown. The organisms were allowed to grow under various conditions of atmosphere and temperature on these slopes. When the chemical substances, including surface active agents, were tested initially, they were incorporated in the agar slopes in the appropriate concentrations.

When bacteriophage activity was tested in the presence of some of the substances used, plates were prepared in exactly the same way as described in a previous paper (Warner, 1950), except that the substances under test were incorporated in the agar medium with which the plates were prepared; loopfuls of the appropriate bacteriophage preparations were then placed on them.

The assessment of the effect of the various modifications and additions to media on bacteriophage activity was based entirely on whether the bacteriophage preparation in question could produce plaques in the higher dilutions of the preparation or not as compared with the same preparation tested on an unmodified medium. Alteration in the clarity of bacteriophage reactions in the presence of additions and modifications to the media, as compared with control experiments, was considered to be an expression of the degree of growth of the organisms, either of the sensitive strain or of the secondarily arising resistant strain, or alternatively, was considered to be related to alterations in intensity of the iridescent phenomenon ; in all these cases bacteriophage reactions will be referred to, non-specifically, as being enhanced or diminished in clarity. The effect of the same addition to or modification of media on the iridescent phenomenon is judged by comparing the amount of iridescence produced in their presence with the amount produced in their absence.

Finally a salt solution described below was used in some experiments.

Further details of materials and methods will be found under the headings of the various experiments.

The Effect of Atmospheric Conditions and Temperature on Iridescence.

Strains No. 22, 30, 46, 52, 108, 130 and 138 all produced iridescence when grown under the following conditions :

1. Aerobically at 37° C. and room temperature.
2. In a moist atmosphere (in a closed airtight tin containing an unplugged test tube of water) at 37° C.
3. In an atmosphere of carbon dioxide (in a closed airtight tin with a test tube containing marble chips and 25 per cent sulphuric acid) at 37° C.

No growth of the organisms was obtained under anaerobic conditions in a McIntosh and Fildes jar.

The Effect of Various Salts on Iridescence.

Strains No. 1, 5, 8, 30, 46, 52, 115, 118 and 138 all produced iridescence, to approximately the same extent as controls without added chemicals when the following were incorporated in the medium of the slopes on which they were grown :

1. Ammonium tartrate	0·5	per cent
2. Sodium glycerophosphate	2	,,
3. Potassium chlorate	8	,,
4. Sodium nitrate	5	,,
5. Gelatin	5	,,
6. Salt (sodium chloride)	5	,,

The Effect of Phenol and Proflavine on the Iridescent Phenomenon of Ps. pyocyanea.

Strains No. 1, 22 and 17 produced iridescence, to the same extent as on control slopes to which nothing had been added, on 2 per cent broth agar slopes containing phenol or proflavine, in concentrations of both 1 : 1000 and 1 : 10,000.

The Effect of a Semi-synthetic Agar Medium on Iridescence and Bacteriophage Action in Ps. pyocyanea.

The following salt solution was added to a 1 per cent agar, with no other nutrient, to the extent of 2 c.c. of salt solution to 50 c.c. of agar.

	g.
Sodium glycerophosphate . . .	6·8
Lactic acid	6·0
Ammonium chloride	2·0
Potassium chloride	1·3
Sodium chloride	11·3
Magnesium sulphate	0·5

Dissolved in 500 c.c. of distilled water.

Then calcium chloride, 2·4 g., was dissolved in a further 50 c.c. of distilled water, which then became cloudy. This solution was filtered and then added to the solution prepared above. The whole was passed through a Seitz filter, and the filtrate transferred to a 4-oz. screw-capped bottle under sterile conditions.

Plates poured from this semi-synthetic agar medium were inoculated with a layer of the same medium seeded with organisms of strains of *Ps. pyocyanea* No. 132 and 138, on to which were placed loopfuls of bacteriophage preparations H/132 and H/138 respectively and tenfold serial dilutions thereof, and were then incubated overnight. A similar pair of plates was prepared in parallel in the same way except that ordinary nutrient agar (1 per cent) was used.

RESULTS.

The results are shown in Table II, from which it can be seen that on nutrient agar strain No. 138 was completely covered with iridescence, so that such bacteriophage reactions as were present were indistinct.

The culture of Strain No. 132 on the same medium showed a moderate amount of iridescence which did not obscure the bacteriophage reactions.

On the semi-synthetic agar medium both strains showed an even opaque white growth with only one or two small patches of iridescence, which may well have been derived from the culture with which the agar was seeded.

From the results of the bacteriophage reactions (Table II) it may be seen that in the case of bacteriophage preparations H/138 tested against Strain No. 138 plaques were obtained in the 10^{-4} dilution on the synthetic agar, whereas on nutrient agar only a very doubtful reaction was obtained in the 10^{-1} dilution.

In the case of the culture of the Strain No. 132, bacteriophage preparation H/132 produced plaques in the 10^{-6} dilution on both media ; on synthetic medium in the high concentration (10^{0} to 10^{-2}) there was no resistant growth in the confluent clearing, and therefore the latter were clearer than those obtained on nutrient agar where the opposite obtained.

Thus it is clear that iridescence was inhibited in both cases, whereas bacteriophage activity was apparently enhanced in one case.

The Effect of Dilution of Broth on the Iridescent Phenomenon and on Bacteriophage Reactions of Ps. pyocyanea.

Agar was added to various dilutions of broth (1 : 2, 1 : 5, and 1 : 10) to the extent of 1 per cent, and each preparation was sterilized in the autoclave. Plates

TABLE II.—*The Effect of a Semi-Synthetic Agar Medium on the Iridescent Phenomenon and*

TABLE II.—*The Effect of a Semi-Synthetic Agar Medium on the Iridescent Phenomenon and Bacteriophage Activity in* Ps. pyocyanea.

Medium.	Bacteriophage preparation.	Organism.	Dilutions. 10^{0}.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	Iridescence.	Degree of growth.
Nutrient agar 1%	H/138	138	C+	C±	—	—	—	—	—	++++	+++
	H/132	132	C+++ M	C+++ M	C+++ M	C++	P+++	P+++	P++	+++	+++
Synthetic medium	H/138	138	C++	C++	C++	P++	P++	P+	P+	±	+++
	H/132	132	C+++	C+++	C+++	C+++	P++++	P++++	P+++	±	+++

This shows the bacteriophage reactions and degree of iridescence on organisms seeded on agar media, one containing ordinary nutrient broth, the other a salt solution. The loopfuls of dilution of the appropriate bacteriophage preparations were then placed on them. The amount of iridescence and the degree of growth was judged by eye.

TABLE III.—*The Effect of Diluting Broth and Adding a Salt Solution to Media on the Iridescent Phenomenon and Bacteriophage Activity on* Ps. pyocyanea.

Broth concentration.	Dilutions of bacteriophage preparation H/138. 10^{0}.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	Growth.	Iridescence.	Dilutions of bacteriophage preparation H/138. 10^{0}.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	Growth.	Iridescence.
Full strength	C+	C+	P++++ ?	—	—	+++	++++	C+++ M	C+++ M	P++++	P++	P+	+++	+
1 : 2	C+++ M	C+++ M	P++++	P++	P+	+++	+++	C+++ M	C+++ M	P+++	P+	P+	+++	++
1 : 5	C+++ M	P++++	P++	P+	P+	++	—	C+++ M	P++++	P+++	P++	P+	+++	—
1 : 10	C+++	P+++	P+++	P++	P+ ?	+	—	C+++ M	C+++ M	P++++	P+	P+	++	—
	Broth media.							Broth and salt solution media.						

This shows the bacteriophage reactions obtained with preparation H/138 tested on cultures of Strain No. 138 on different modifications (see text) of agar media.

were poured with the three different nutrient media thus obtained, and also with ordinary nutrient agar (1 per cent). The plates of all four media, which contained different concentrations of broth, were then inoculated with a seeded layer of organisms of Strain No. 138, the seeded layer consisting of the same medium as the plate on to which it was poured.

Bacteriophage preparation H/138 was titrated, and loopfuls of it and of the dilutions were placed on the plates prepared above.

Results.

The results are shown on the left-hand side of Table III, but will be described with those of the next experiment, with which this was done in parallel.

The Effect of Diluted Broth Media with added Salt Solution on Iridescence and Bacteriophage Activity in Ps. pyocyanea.

The same medium was prepared as in the last experiment, except that to each 50 c.c. of it 2 c.c. of the salt solution described in this paper was added. Subsequently precisely the same procedure was adopted as in the last experiment.

Results.

On examination of the left-hand side of Table III it is seen that, in the first place, dilution of broth to 1 part in 5 completely inhibited the iridescence of Strain No. 138, and in fact it was diminished even when diluted to 1 part in 2. Secondly, bacteriophage action was considerably enhanced on the medium containing broth diluted to 1 in 2, and even on that containing the broth diluted to 1 in 10 was still plainly visible.

The salt solution when added to the full strength broth medium inhibited iridescence markedly (right-hand side Table III), and also enhanced bacteriophage activity to the same degree as on the diluted broth media without salt solution.

Finally it is seen that the salt solution increased bacterial growth when added to the 1 in 5 and the 1 in 10 diluted broth media ; with the former concentration the growth obtained with salt solution was equal to that on full strength broth agar, either with or without salt solution.

To summarize, the modifications of the media described here inhibited iridescence completely in two cases, and in one case enhanced the bacteriophage action. In the other case the latter has not been affected. Addition of salt solution to diluted broth agar apparently increased the growth of organisms.

The Effect of Surface Active Agents on the Iridescent Phenomenon of Ps. pyocyanea.

Various surface active agents were added to 2 per cent broth agar in various concentrations. Slopes prepared from these were inoculated with organisms of different strains of *Ps. pyocyanea*. Control slopes containing no surface active agents were also inoculated with the organisms of the same strains. The slopes were incubated overnight and examined the following day for iridescence. The results are shown in Table IV.

TABLE IV.

Surface active agents.	Concentration (per cent.).	Strains of *Ps. pyocyanea* in which iridescence inhibited.
Teepol	1	125, 23, 30, 46, 112, 119, 14.
Hexyl-resorcinol	0·1	125, 23, 30, 46, 112, 119, 22
Sodium desoxycholate	1	125, 55, 23, 30, 46, 119, 22, 54, 76, 129, 132
Sodium lauryl sulphate	2	125, 23, 30, 46, 119, 76.
Saponin (white)	1	125, 22, 14.
,, ,,	5	125, 55, 30, 22, 14, 54, 129, 132, 131, 109, 138, 29, 3, 139, 7.
,, (brown)	1	125, 22, 14.
Crill No. 1	1	125, 55.
,, No. 31	1	125, 55.
,, No. 8	10	125, 46, 119.
Cetavlon	0·1	125, 55, 30, 46, 29, 3.
,,	0·075	125, 55, 30, 46, 112, 54, 138, 29, 3.

As a result of the above experiments, two of the surface active agents, namely white saponin and sodium desoxycholate, were chosen and incorporated in a nutrient agar medium in suitable concentrations. Using plates made from these media bacteriophage titrations were performed on organisms of sensitive strains of *Ps. pyocyanea*. Control plates without surface active agent were prepared in the same way at the same time. The titre of bacteriophage and the amount of iridescence were recorded and the results are shown in Tables V and VI.

Results.

Table IV shows the surface active agents that were tested. Although they were tested in several concentrations, only those which did not inhibit growth and did inhibit iridescence are shown. All the surface active agents had some inhibitory activity in the concentrations recorded on all strains tested, but " Cetavlon " and Crill No. 8 would not abolish it completely in any concentration on those strains that were tested. However, from the results it is obvious that a representative sample of surface active agents have a marked effect on the iridescence produced by a number of different strains of *Ps. pyocyanea*. Each one of 5 of the surface active agents (Teepol, hexyl-resorcinol, sodium desoxycholate, sodium lauryl sulphate, white saponin and " Cetavlon ") di persed iridescence produced by six different strains of the organism.

The purpose of this part of the experiment was to discover a surface active agent which dispersed iridescence and had a minimal effect on the growth of the strain of *Ps. pyocyanea* being tested. Of all the agents tried, white saponin and sodium desoxycholate had the greatest differences in the concentrations required to disperse iridescence, and those which would visibly inhibit growth of the organisms. These agents were, therefore, tested against various bacteriophage preparations and iridescence on the same cultures. The effect of white saponin is shown in Table V. This shows that the iridescence produced by 15 strains of organism (1–15, Table V) is dispersed by the saponin without significantly diminishing bacteriophage activity except perhaps with No. 3 (bacteriophage preparation No. L/54 on *Ps. pyocyanea* Strain No. 54) and No. 9 (preparation

TABLE V.—*Effect of Incorporating 5 per cent Saponin in Nutrient Agar on Iridescence and Bacteriophages.*

	Organism.	Bacteriophage.	Plain 1 per cent nutrient agar. Titre.		Iridescence.	1 per cent nutrient agar + 5 per cent saponin. Titre.		Iridescence.
1	109	L/109	10^0 C+	..	+++	10^0 C+++	10^{-2} P+++	—
2	22	H/22	10^0 C++	10^{-4} P++	+++	10^0 C++	10^{-4} P++	—
3	54	L/54	10^0 C++	10^{-2} P+++	+++	10^0 C+++	10^{-4} P++	—
4	129	L/129	10^0 C+++	10^{-3} P+	+++	10^0 C++++	10^{-4} P+	—
5	138	H/138	10^0 C+++	10^{-4} P+++	+++	10^0 C+++	10^{-4} P+++	—
6	29	B/29	10^0 C+++	10^{-4} P++	+++	10^0 C+++	10^{-4} P++	—
7	125	H/125	10^0 C+++	10^{-4} P+	++	10^0 C++++	10^{-4} P+	—
8	131	53/1/131	10^0 C+++	10^{-4} P+	++	10^0 C++++	10^{-4} P++	—
9	14	H/132/14	10^0 C+++	10^{-2} P++	++	10^0 C++++	10^{-1} P+++	—
10	3	30/3	10^0 C+++	10^{-4} P+++	++	10^0 C+++	10^{-4} P+	—
11	30	H/30	10^0 C++	10^{-4} P+++	++	10^0 C+++	10^{-4} P+++	—
12	55	H/125/55	10^0 C++++	10^{-4} P+++	++	10^0 C++++	10^{-4} P+++	—
13	132	H/132	10^0 C+++	10^{-4} P+++	++	10^0 C+++	10^{-4} P+++	—
14	139	J/139	10^0 C+++	10^{-4} P++	+	10^0 C+++	10^{-4} P++	—
15	7	A/2/7	10^0 C++	10^{-4} P+++	+	10^0 C++	10^{-4} P++++	—
16	10	P1/10	10^0 C+++	10^{-4} P+++	—	10^0 C+++	10^{-4} P++	—
17	16	H/132/16	10^0 C+++	..	—	..	10^0 P+++	—
18	4	H/4	10^0 C+++	10^{-4}	—	10^0 C+++	10^{-4} P++++	—

+++ = Confluent iridescence. ++ = Iridescent patches (numerous). + = Few iridescent patches.

TABLE VI.—*Effect of Incorporating* 1 *per cent Sodium Desoxycholate in Nutrient Agar on Iridescence and Bacteriophage.*

Organism.	Bacteriophage.	Plain 1 per cent nutrient agar.			1 per cent nutrient agar + 1 per cent sodium desoxycholate.		
		Titre.		Iridescence.	Titre.		Iridescence.
22	H/22	10^0 C++	10^{-4} P+++	+++	10^0 C++	10^{-3} P+++	—
129	L/129	10^0 C+++	10^{-4} P+	++	10^0 C+++	10^{-4} P+	—
132	H/132	10^0 C+++	10^{-4} P++	+++	10^0 C++++	10^{-4} P+++	—
54	L/54	10^0 C++	10^{-3} C+	++	10^0 C+++	10^{-4} C+	—
55	H/125/55	10^0 C+++	10^{-4} C+++	+	10^0 C+++	10^{-4} C+++	—

H/132/14 on Strain No. 14). On the other hand, No. 1 (preparation L/109 on Strain No. 109) and No. 4 (preparation No. L/129 on Strain No. 129) were definitely enhanced.

Saponin has no effect on bacteriophages of No. 16, 17 and 18 (preparation No. P1/10, H/132/16 and H/4).

The bacteriophage preparations shown in Table V are active against 31 different strains of *Ps. pyocyanea* out of 65 tested (Warner, 1950), that is to say about 48 per cent of all strains.

Table VI shows the effect of incorporating 1 per cent sodium desoxycholate in the agar medium on iridescence and bacteriophage in 5 cases. It is clear from this table that the surface active agent has abolished iridescence in every case, whereas it has not affected bacteriophage reactions in any case.

DISCUSSION.

No observations connected with the iridescent phenomenon seen in *Ps. pyocyanea* have been found in a survey of the available literature on this subject since the time of, and including, Hadley's first paper (1924), which disagree with one another or with those described in this paper. Thus certain facts may be regarded as well established, although the conclusions drawn from them fall into one of two mutually contradictory groups. The first is that the iridescent phenomenon is caused by bacteriophage, and the second that it is not.

The visible properties and behaviour of the iridescent phenomenon when contrasted with bacteriophage plaques (Table I) show more differences than similarities. Thus, at the outset, bacteriophage activity differs from the lysis associated with iridescence.

In order to explain these differences and in order to identify the two phenomena Hadley (1924) suggests, and is supported by Burnet (1930), that the iridescent clearings are examples of slow-speed bacteriophage activity. If this were the case then, since comparatively large masses of bacteria are lysed in the iridescent clearings, there should be a high concentration of bacteriophage particles, or at least higher than elsewhere in such cultures, in those particular areas. That there is a higher concentration of bacteriophage particles in the iridescent clearings is not borne out by experiments performed by Hadley (1924) in which he touched an iridescent patch in a culture of *Ps. pyocyanea* and subsequently touched a colony, which did not show any iridescence, in the same culture, without inducing an iridescent clearing in the colony. This result has also been confirmed by the present author.

No theories have been found in the literature, apart from the above, which attempt to explain away the more obvious differences between iridescent clearings and bacteriophage action (Table I), and which identify the latter as the cause of the former in spite of those differences.

There are five possible ways in which bacteriophage could be incriminated as the causal agent of the iridescent phenomenon, and these are as follows :

1. That iridescence is a phenomenon accompanying bacteriophage action on *Ps. pyocyanea*.

This can be dismissed, since no iridescence has been noted with any bacteriophage of *Ps. pyocyanea*.

2. That iridescence accompanies lysogenesis in *Ps. pyocyanea*.

This can be dismissed because non-iridescent strains of *Ps. pyocyanea* may be lysogenic.

3. That iridescence is associated with special strains of either one or of both bacteriophage and *Ps. pyocyanea*.

If this is so these strains must appear fairly commonly, since iridescence is associated with about one-half of all strains of *Ps. pyocyanea*. It would be surprising, then, if such special strains or combination of strains had not been encountered several times during the isolation of bacteriophages. They were not encountered during the isolation of 19 bacteriophage preparations which were active against more than one-half of a series of 65 strains of *Ps. pyocyanea* (Warner, 1950).

4. That iridescence accompanies lysogenesis, but is associated with special strains of either one or of both bacteriophage and *Ps. pyocyanea*.

Again such special strains or combination of strains ought to be fairly common, since, as mentioned above, about 50 per cent of all strains of *Ps. pyocyanea* show iridescence. In addition to this very few of the secondarily arising resistant growths found in the centre of confluent clearings showed the iridescent phenomenon, and only did so on some of the occasions when the parent growth showed iridescence. If this fourth possibility were true then about one-half of the secondarily arising resistant growths should have shown iridescence irrespective of whether the parent growth exhibited it or not.

5. That iridescence is the result of a special relationship brought about by an unknown circumstance between bacteriophage and bacterium in a lysogenic strain.

This assumes a type of behaviour which has not been known to accompany bacteriophage activity before, and which is not explained by the established facts that are, at present, known about bacteriophage. However, it is the only way in which the iridescent phenomenon can be linked with bacteriophage action. In order to maintain this hypothesis it must be accepted that two surface active agents, differing chemically from one another and acting on the growth of neither the bacteria nor the bacteriophage, interfere specifically with this special virus-host relationship. However a simpler explanation, without invoking bacteriophage, would be that the iridescent material itself causes lysis (because lysis does not occur in the absence of the iridescent material) and that, as would be expected from its physical properties, surface active agents disperse it in a non-specific manner.

To this evidence can be added the results of experiments here reported, which show that—

(*a*) the use of a semi-synthetic agar medium (Table II),
(*b*) the dilution of broth in an agar medium (Table III),
(*c*) the addition of a salt solution to broth agar medium

will inhibit iridescence but does not affect bacteriophage activity in two cases, and in the absence of the iridescence the bacteriophage reaction appeared stronger and clearer with one strain of *Ps. pyocyanea* (Table III).

To summarize, there is no evidence that bacteriophages have any causal relationship with the spontaneously occurring iridescent lytic areas found on surface cultures of *Ps. pyocyanea*. Every observation described here and those reliable ones in the literature when considered together suggest to the point of

virtual proof that bacteriophage activity and the iridescent clearings are two distinct entities.

As to the nature of the iridescent clearings in *Ps. pyocyanea*, the most plausible theory is that it is an expression of bacterial variation as follows: Within a surface culture of *Ps. pyocyanea* a variant colony arises which produces an oily or waxy metabolite. The metabolite then lyses the growth of the parent strain.

This theory can be made, without straining possibilities, to fit most of the facts. Firstly it is in accord with the ease with which *Ps. pyocyanea* undergoes spontaneous variation; that bacterial variation occurs readily in *Ps. pyocyanea* is contended by Hadley (1927 and 1928). Secondly, if the iridescent material, as it spreads, is accompanied by lysis of the underlying culture to the extent to which it has spread, then it is reasonable to suspect that the material has caused lysis.

Thirdly, the material has some of the properties of a fatty acid, and could possibly be part of, or the whole of, pyocyanase (Albert, 1948, personal communication) of the older workers, which is known to lyse bacteria (Emmerich and Low, 1899), and which has been shown *in vitro* to consist of an active free fatty acid dissolved in inactive neutral fats and phosphatides (Hettche, 1934; Hettche and Vogel, 1937). Schoental (1941) obtained an oily chloroform-soluble substance with bacteriolytic properties from old broth cultures on *Ps. pyocyanea*. It is possible that all three of these substances contain the same active lytic principle.

Finally the theory could feasibly account for the visible properties of the iridescent clearings and the iridescent haloes seen in surface cultures of *Ps. pyocyanea*.

This explanation may not be the true one, but it suggests a profitable line of research, namely, the investigation of the properties, chemical and biological, of the iridescent material.

SUMMARY.

The important visible properties of iridescent clearings in *Ps. pyocyanea* are described and contrasted with bacteriophage plaques, showing that these two phenomena have only two characteristics in common, namely, lysis of organisms, and sometimes a superficial resemblance to one another.

The iridescent material was separated from cultures of *Ps. pyocyanea*, and was found to be a waxy and fatty or oily substance, brownish in colour, and easily moulded.

The production of iridescent material was inhibited in surface cultures of *Ps. pyocyanea*—

(*a*) By using diluted broth in the preparation of the nutrient agar medium.

(*b*) By using a semi-synthetic agar medium.

(*c*) By adding a salt solution to the medium.

(*d*) By adding surface active agents in suitable concentrations to the medium.

Saponin, in concentrations which disperse iridescence, has no effect on a series of different bacteriophages, which attack over one-half of a group of 65 strains of *Ps. pyocyanea*. One per cent sodium desoxycholate is shown to have

similar properties, but was tested with only five bacteriophages with a restricted range of activity.

The results are discussed, and it is concluded that there is no evidence for the assumption that bacteriophage is the cause of the iridescent clearings and that the evidence against it is considerable.

Bacteriophage activity and iridescent clearing in cultures of *Ps. pyocyanea* appear to be two distinct phenomena ; the latter is probably the result of bacterial variation.

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THE TOXICITY OF A PRODUCT OF REACTION OF MUSTARD GAS WITH SERUM PROTEINS.

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THE possibility of chemical reaction between mustard gas and proteins was investigated simultaneously by a number of teams working for the Ministry of Supply between 1939 and 1944. Immunological evidence for such a reaction had already been put forward by Berenblum and Wormall (1939). Subsequently, Collumbine and Rydon (1946) isolated leucotaxine from the products after horse serum and mustard gas had been incubated together at 37° C., and Pirie (1947) demonstrated a reaction product from ox cornea collagen and mustard gas. Studies of the effects of mustard gas on enzyme systems led Peters and his collaborators (Peters, 1947) to suggest possible points of attack of mustard gas on a protein molecule, and Crane and Rydon (1947) discussed in detail the chemistry of the formation of mustard gas—protein compounds. In the course of experiments on the effects of mustard gas on living cells (Fell and Allsopp, 1948), we made a number of semi-quantitative observations which confirmed those of the other workers. They were reported in detail to the Ministry of Supply (Allsopp and Fell, 1941, 1943, 1944), and need not be recapitulated. The present paper is concerned mainly with an investigation of the toxicity of a product of reaction of mustard gas with serum protein. The results of one chemical investigation which was made in order that the biological experiments might be properly controlled are also included.

PREPARATION OF MATERIALS.

Mustard gas and fowl plasma were allowed to react by placing pure redistilled ββ′-dichlordiethyl sulphide with plasma in a closed tube. The mixture was stirred continuously for 24 hours at 37° C.; no attempt was made to continue the reaction until all the mustard gas was used up. These proportions of the reagents represented a large excess of mustard gas when compared with those used in work on enzymes (Dixon and Needham, 1946; Peters, 1947), but our conditions resembled those in the experiments of Collumbine and Rydon, and of Pirie, except that no attempt was made to control the pH of the mixture; they appeared to us to approximate more nearly to the conditions which might obtain *immediately* under a drop of mustard gas in contact with tissue, i.e. where the cells are almost instantaneously coagulated.

Analysis of the reaction mixture for unchanged mustard gas.

Before the contents of the reaction vessel were prepared for biological tests, it was desirable to know how much unchanged mustard gas they contained. For this purpose an attempt was made to aspirate all the unchanged mustard gas from the reaction vessel, and to estimate it by an absorptiometric modification of the usual colorimetric method based on its reaction with sodium iodoplatinate and starch (Allsopp, 1950). The iodoplatinate, of course, also reacts with thiodiglycol. For convenience, therefore, the volatile material so estimated is described as " mustard gas," while the non-volatile substance remaining in the reaction vessel, which could also be estimated, is described as " thiodiglycol."

Various proportions of mustard gas and plasma were taken in the reaction vessel, which after 24 hours stirring was connected to a train of seven absorption vessels containing in all 100 c.c. of 5 per cent. acetic acid. Air was drawn through at the rate of 100 c.c. per minute for six hours, during which the reaction vessel was kept at 37° to 40° C. Frothing was prevented by the addition of a small measured quantity of caprylic acid to the contents, allowance for this being made subsequently. The " free " mustard gas was then estimated in the acetic acid from the absorption vessels. The residue was passed through an ordinary filter paper in order to remove the bulk of the coarse precipitate which control experiments showed might interfere with the analysis by producing a turbidity. The precipitate was carefully washed with successive portions of distilled water, to recover thiodiglycol, until the total volume of the filtrate was 19 c.c., and 1 c.c. of glacial acetic acid was added. The total volume was made up to 100 c.c., i.e. equal to that in the aspiration bottles, with 5 per cent acetic acid.

These solutions were again diluted with 5 per cent acetic acid until they could be expected to contain the equivalent of 0·025 mg. mustard gas per c.c., this concentration having been found to be most satisfactory in the absorptiometer before the estimations were made. Each estimation was controlled by an experiment in which the same weight of mustard gas was incubated with Pannett and Compton's saline at 37° for 24 hours. The weights of " mustard gas " and " thiodiglycol " recovered under identical conditions in the control experiment were taken as standard, and those recovered from the plasma were calculated as percentages of them.

TABLE I.—*Recovery of Mustard Gas from the Products of its Interaction with Fowl Plasma.*

Total volume of plasma.	Initial weight of mustard gas.	Weight of mustard gas per c.c. of plasma.	Mustard gas recovered		Total " loss " of mustard gas.	" Loss " per c.c. of plasma.
			By Aspiration.	As "Thiodiglycol."		
c.c.	mg.	mg./c.c.	mg.	mg.	mg.	mg./c.c.
8	131·1	16·4	0·03	125·20	5·87	0·74
4	45·3	11·3	0·02	41·93	3·35	0·84
12	60·2	5·0	0·06	50·39	9·75	0·81
8	25·8	3·2	18·14		7·66	0·96

The results of a series of such analyses are recorded in Table I, which shows that, under the conditions described, the weight of mustard gas per c.c. of plasma which could not be recovered from the reaction vessel was independent of the initial proportions of agent and plasma, and appeared to be constant, at least

within the limited accuracy attainable by the analytical method. This result is to be expected if a chemical reaction had taken place.

Table I also revealed that at the higher concentrations used in preparing specimens for the toxicity tests (see below) considerable quantities of unchanged mustard gas would remain. The following method for its removal, together with any ionizable products which might be toxic, was therefore adopted.

Extraction of unchanged mustard gas from the reaction mixture.

The mixture in the reaction vessel was ground to a smooth paste with a glass rod and was then spread in a shallow layer in a Petri dish and left to stand over P_2O_5 and absorbent charcoal for four weeks in a vacuum desiccator evacuated by a " Highvac " pump running continuously. The dry solid obtained could be ground to a powder, off-white in colour, which was rather hygroscopic and began to smell of mustard gas when exposed to moist air. It was therefore extracted with boiling acetone in a Soxhlet apparatus for 16 hours, and portions were also dialysed through collodion membranes against frequent changes of distilled water for at least 14 days, i.e. for some time after the water ceased to give any reaction for chlorine.

By the kindness of Professor Sir Ian Heilbron, some of the powdered reaction product, after it had been washed several times with ether, was submitted to micro-analysis for chlorine and sulphur in his laboratory. The atomic ratio was Cl : S :: 2·2 : 1 as compared with 6 : 1 in untreated plasma. The treated plasma thus contained an excess of sulphur. An increase in S-content in protein treated with mustard gas was also recorded by Wormall (Banks, Boursnell, Francis, Hopwood and Wormall, 1946).

TOXICITY TESTS.

The starting materials were one part by volume of pure redistilled ββ′-dichlordiethyl sulphide incubated at 37° C. for 24 hours, with continuous stirring, in 20 volumes of serum. Identical results were obtained with fowl plasma, fowl serum and human serum, and only typical experiments with human serum need therefore be described.

Specimens were prepared for tests as follows:

α. The contents of the reaction vessel were dried and washed with acetone as described above. The powder was yellowish-grey in colour.

β. Some of α was ground in distilled water and dialysed. The final powder was yellow.

γ. In this case the contents of the reaction vessel were dialysed before drying and extraction with acetone. The product resembled α in appearance.

δ (control). Part of the same human serum was dried and extracted in the same way as α.

ε (control). Part of δ was dialysed under the same conditions as β. Both δ and ε were orange in colour.

Biological technique.

Each substance was tested on six mice by subcutaneous inoculation, under ether anaesthesia, into the upper part of the thigh. The dose for each animal was about 3·4 mg.

The skin was closely shaved, and beneath it a small pocket was made with fine scissors. The desiccated material to be noculated was damped with distilled water sufficiently to make the particles adhere, and was then collected on the tip of a cataract knife and inserted into the pocket of skin, the mouth of which was sealed with collodion.

For histological examination, the lesions were removed and fixed in 3 per cent acetic Zenker's fluid ; sections were stained with Ehrlich's haematoxylin and erythrosine or with azan.

RESULTS.

Superficial examination.

Four days after inoculation, three lesions in each of the controls δ and ε were healed ; the remaining animals still had small scabs. The wound in set α bore scabs comparable with these, but one was much more inflamed. In sets β and γ, which were comparable in severity, all the lesions bore scabs, were inflamed, and appeared not to be healing. Subsequently, control sets δ and ε made steady progress and healed completely. Set β appeared to be healing at first ; on the sixth day, the superficial appearance was little different from that of the controls, but thereafter the lesions made little progress. Set α healed very slowly, with large scabs which sloughed repeatedly, while the appearance of set γ was rather worse than that of set β, but better than that of set α throughout the later stages.

Half the animals in each set were killed on the 11th day after inoculation, and the remainder on the 12th day, and the lesions were excised. In control sets δ and ε, it was difficult to detect the site of the initial incision ; at most, only a faint scar was visible. Set β showed only slight scars or small scabs with some adhesion of epidermis to muscle. The wounds tended to re-open under tension. In set γ the scabs were rather larger, but the adhesions were comparable, while set α had large scabs and thick adhesions.

Histological examinations.

Set δ (control). All the lesions were fully epithelialized and there was little scar tissue. Some leucocytic infiltration was visible, but there were no signs of unresorbed serum.

Set ε (control). Four of the specimens were comparable with those of set δ, but two contained a pocket of pus.

Set α (undialysed reaction product). Both epidermis and dermis above the pockets were necrotic, and the pockets were filled with unresorbed serum. The skin and serum were being sloughed, and the epidermis was growing down the under surface of the pockets. There was much leucocytic infiltration but not much pus was visible.

Set β (dialysed reaction product). The appearance generally was the same as in set α, but the lesion was less severe. The overlying tissue was less necrotic.

Set γ (dialysed reaction product). These specimens were little different from set α, but the lesions were not quite so severe.

DISCUSSION.

The biological tests leave no doubt that the products of the reaction of mustard gas with human serum proteins, as prepared in the present experiments, contained a substance which was sufficiently toxic to retard the healing of super-

ficial skin wounds. We do not think that the effect was due to contamination with mustard gas itself, in view of the care taken to remove it, especially in specimens β and γ. There is the possibility that mustard gas might slowly be regenerated from an addition compound under *in vivo* conditions (Crane and Rydon, 1947); such substances were more likely to be present in the undialysed specimen α than in β and γ, both of which caused only a slightly less severe aspect in the lesions both superficially and histologically. The toxicity of the dialysed materials was so small and so localized that it is unlikely to have any direct connection with vesication; this result would appear also to exclude leucotaxine as the active agent.

The observation that these substances were not resorbed in tissue and were sloughed suggests that they might persist in the area of fixed tissue immediately beneath the site of contact of liquid mustard gas and human skin, an area which personal experience shows sometimes heals very slowly and whose location can still be seen several years after the original lesion was apparently healed. The experiments which have been described emphasize once again the variations in the behaviour of mustard gas as a toxic agent and the necessity for still closer examination of its effects on skin.

SUMMARY.

Evidence is adduced to show that, when mustard gas and human serum were incubated together for 24 hours at 37° C., a substance remained in the products, after rigorous precautions to remove unchanged mustard gas, which was sufficiently toxic to impair the healing of skin lesions in mice.

The work described formed part of a programme of research carried out at the Strangeways Laboratory between 1939 and 1944 for the Department of Chemical Defence, Ministry of Supply. We are indebted to the Chief Scientist, Ministry of Supply, for permission to publish the results.

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